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Docetaxel Regulates the Interaction of p53 with MDM2 and Sin3A to Suppress MCF-7 Breast Cancer Cells

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Abstract

Docetaxel is one of the most actively used chemotherapeutic agent in breast cancer which is the most frequent tumor in women. Recent studies propose that blocking the p53-MDM2 interaction may be effective in cancer treatment while the Sin3A mutation enhances cell proliferation in estrogen receptor (ER)-positive breast cancers. We aimed to investigate the effects of docetaxel on gene expression interactions and apoptosis in ER-positive breast cancer cell lines (MCF-7). MCF-7 cells were incubated for 24h with the treatment of escalating molar concentrations of docetaxel. The p53, MDM2 and Sin3A gene expression levels were measured by Real-Time PCR. The MTT assay was used to determine cellular viability. Apoptotic cells were detected by TUNEL. The mRNA expressions of p53, MDM2, and Sin3A increased in the same dose-dependent manner suggesting the highest effective level is 100nM docetaxel concentration ($p<0.001$). The p53 expression levels were strongly correlated with MDM2 ($r=0.9379$; $p<10^{-7}$) and Sin3A ($r=0.9965$; $p<10^{-13}$) in untreated, 10nM, 100nM and 1µM docetaxel concentrations. Cell viability of MCF-7 cells decreased dramatically in the 10µM and 100µM docetaxel treatments ($p<0.001$) and the IC_{50} value was 10µM. Apoptotic cell density was enhanced with the treatments of 10nM, 100nM, and 1µM docetaxel ($p<0.001$) in response to the gene expression levels. Our findings suggest that docetaxel directs the MCF-7 breast cancer cells to apoptosis in a dose-dependent manner and may thus further regulate the interaction of tumor suppressor p53 expression, protecting it from MDM2-mediated degradation and inhibiting Sin3A-mediated cell proliferation in compliance with the apoptotic cell density.

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1. INTRODUCTION

Docetaxel, used as an effective chemotherapeutic agent in breast cancer, binds to β-tubulins and inhibits the depolymerization of microtubulins, causing cell cycle arrest and apoptosis (McGrogan *et al.*, 2008). It causes apoptosis in different ways, either directly or indirectly. The main therapeutic effect of docetaxel is the suppression of microtubule dynamics (assembly and separation), cell cycle disruption, and phosphorylation of Bcl-2 (Dumontet and Jordan, 2010). In a recent study, docetaxel at a dose of 100 mg/m^2 has been found to be effective in patients with advanced or recurrent breast cancer with supportive therapies (Hirata *et al.*, 2021). The tumor suppressor gene TP53, encoding the p53 protein, is one of the most common mutated gene in breast cancer. The tumor suppressor p53 is a pivotal regulator in controlling cell proliferation and death in response to potentially oncogenic conditions, and therefore, the

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interactors of p53-family proteins unveil regulatory elements that could be targeted more efficient use of drugs for cancer treatment (Collavin *et al.,* 2010).

The p53, a member of the tumor suppressor gene family, is the most widely studied gene in cancer research. Cellular pathways of p53 mediate tumor suppression resulting in either cellular death or the maintenance of cellular homeostasis. Recent knowledge indicates that cells can activate p53 signaling through positive and negative regulators of p53 having a critical function in cell cycle, apoptosis, senescence, stem cell differentiation, metabolism, DNA repair, ROS, and mitochondria in response to specific stress or a combination of stresses (Levine, 2020). The MDM2 protein encoded by the MDM2 gene can form a complex with the p53 tumor suppressor protein and prevents the apoptosis effect of p53 by keeping p53 under control (Freedman *et al.,* 1999). It is an oncogene-functional intracellular protein that is thought to be a significant target for cancer survival. It stimulates cell survival and growth while arresting the coordinated cell cycle and suppressing apoptosis (Vazquez *et al.,* 2008). The oncogene MDM2 might be an independent negative prognostic marker in breast carcinomas (Turbin *et al.,* 2006). Furthermore, current studies proposed that inhibitors designed to block the MDM2–p53 interaction may be effective in the treatment of human cancer by reactivating the tumor suppressor gene p53 (Wang *et al.,* 2017; Konopleva *et al.,* 2020).

Transcriptional regulator switch-independent 3 family member A (Sin3A) is essential for embryogenesis and T-Cell development and is required for the development and homeostasis of cells in the lymphoid lineage (Cowley *et al.,* 2005). Sin3A may also play an important role in regulating homeostasis and development, including cell death and mitochondrial biogenesis (Pile *et al.,* 2003). Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3A (Murphy *et al.,* 1999). The Sin3A gene has been studied for more than two decades in cancer research and is known to be activated in several tumors mediated by interactions with p53, HDAC1/2, Mad, MeCP2, LSD1, REST, NRSF, CTCF, STAT3, and ERα (Murphy *et al.,* 1999; Zilfou *et al.,* 2001; Ellison-Zelski and Alarid, 2010; Watanabe *et al.,* 2018; Yang *et al.,* 2018; Gambi *et al.,* 2019; Zhao *et al.,* 2019; Jayaprakash *et al.,* 2021). Sin3A is a pro-survival protein that promotes growth of estrogen receptor (ER) positive breast cancer cells by preventing apoptosis through the repression of key proapoptotic genes. The Sin3A may therefore be an effective chemotherapeutic target in controlling the survival and growth of ERα-positive tumors (Ellison-Zelski and Alarid, 2010). The Sin3A is critical in the cellular function of cellular proliferation, differentiation, apoptosis, and cell cycle regulation (Kadamb *et al.,* 2013). The role of Sin3A in oncogenic potential is proposed to be associated with the transcriptional repression of tumor suppressor genes (Gambi *et al.,* 2019).

Sin3A mutation enhances MCF-7 cell proliferation through ERα expression and loses its transcriptional repression function due to its cytoplasmic localization. The reduction in Sin3A may also cause the recurrence of ERα-positive breast cancers (Watanabe *et al.,* 2018). Furthermore, the p53-mediated apoptosis in MCF-7 cells is regulated by very complex and diverse mechanisms and negatively regulated by HDAC3–ERα in a caspase-7-dependent manner (Park *et al.,* 2020). It is obvious that breast cancer is controlled by the expressions of a number of cancer-related genes including p53, MDM2, and Sin3A. Therefore, the function and the interaction of suppressive and oncogenic genes provide potential targets for further evaluation of chemotherapy in breast cancer cells. In this research, we aimed to investigate the effects of docetaxel as an actively used chemotherapeutic agent on p53, MDM2, and Sin3A and their interactions in MCF-7 estrogen receptor-positive breast cancer cells. The cell viability measurement and apoptotic cell assay facilitated to evaluation of the function of gene expressions in MCF-7 cells incubated for 24 h and treated with different molar concentrations of docetaxel in vitro.

2. MATERIAL and METHODS

2.1. Cell Culture, Passaging of Cells, and Cell Count

The MCF-7 cell line (11. passage) was commercially available from the FMD Institute (BioSample: SAMN02054478). MCF-7 cell lines were cultured in EMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) solution. Cultures were incubated at 37 °C with 5% carbon dioxide (CO_2) and 95% humidity. Cell culture studies were performed in the Bio Safety Cabinet (Class II). The medium of the cells that reached certain confluency (80%) was removed and washed with 4-5 mL PBS buffer. Subsequently, 3 mL trypsin/EDTA (Ethylene Diamine Tetraacetic Acid) was added. They were incubated in a CO_2 incubator during the period. 8 mL of fresh medium was then added and centrifuged for 5 min at 800 x g. The supernatant part was removed from the centrifuged cell suspension and 10 mL of fresh medium was added to the pellet. These passaged cell cultures were left to incubation at 37 ^{0}C in a 5% CO_2 incubator containing 95% humidity. 1µL of the cell suspension to be counted was transferred to a 0.5 mL eppendorf tube and 90 µL trypan blue dye was added to it and 10 µL was transferred to both chambers of the hemocytometer and cell count was performed under an inverted microscope. Cell concentration was determined using the formula (1) below.

$$\text{Number of cell /mL} = \text{Average number of cells in chambers} \times DF \times 10^4 \quad (1)$$

DF: Dilution factor.

10^4: Factor arising from chamber sizes on the slide (1 mm^3).

Thus, $5x10^6$ cells were transferred to each 100 mm^2 culture petri dish and 5000 cells to each well of the 96-well culture plate.

2.2. Drug Treatment

Docetaxel drug (Taxotere, Rhone-Poulenc Rorer Pharmaceutical) was used to treat the duplicate of each sample in wells at various concentrations. Docetaxel was diluted by the growth media, and then MCF-7 cells were exposed to the growth medium supplemented with concentrations of 10 nM, 100 nM, 1 µM, 10 µM, and 100 µM and 200µL treated to each well with equal numbers of cells, correspondingly. They were incubated for 24 h at 37°C, 5% CO_2.

2.3. Cell Viability Measurement

The cytotoxic activities of the samples on existing cell lines were measured using the 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) kit (Cell Proliferation MTT Kit, Roche, Cat No: 11465007001) according to the manufacturer's instructions. The water-insoluble MTT tetrazolium salt is reduced to the water-soluble orange-colored formazan compound by the mitochondrial enzyme activity in metabolically active cells, and the product was measured in a microplate reader at 450 nm. The percent cell cytotoxicity/viability curve was drawn against the applied dose, and the 50% suppressive concentration (IC_{50}) value was calculated with the logarithmic slope graph in Microsoft Excel Program.

2.4. Histochemical Staining and TUNEL Assay

Inoculations were carried out in equal amounts (approximately 5000 cells suspended in 100 µL medium) on the upper surfaces of the lam in each petri dish. Docetaxel treatments were applied after the cells adhered to the lam. Following the end of the 24 h incubation period, PBS was added and cell washing was performed after removing the medium in the petri dish. The cells adhered to the lam were fixed with methanol at -20 ^{0}C for 10 min. The lams were washed with PBS and dried.

Terminal deoxynucleotidyl transferase Mediated Bio-dUTP Nick end Labeling (TUNEL) was used for histochemical detection of apoptosis. The TUNEL kit (Roche, In Situ Cell Death

Detection Kit, TMR red, Cat No: 12156792910) was used for the staining process. Adherent cell staining was performed according to the experimental protocol included in the kit. The "Stereological Optic Fractionator Frame" method was used to compare the results of TUNEL staining between the groups. This assay indicates late-stage apoptosis. The assay was performed under the stereology workstation system (BioPrecision MAC 5000 controller system, Ludl Electronic Products, Hawthorne, NY) and stereology software (Stereo Investigator version 9.0, Microbrightfield, Rochester, VT) under a light microscope with attachment (Leica, Cambridge, UK). For the determination of apoptotic cells on MCF-7 cell preparations, the method of "Unident Counting Frame and Fractionator" was used and the TUNEL positive cell density in each preparation belonging to all groups was calculated according to the following formula (2):

$$PCD = PCN / (FA \times FN) \quad (2)$$

PCD: TUNEL positive cell density per μm^2 area

PCN: TUNEL positive cell count

FA: Frame area (μm^2)

FN: The number of frames

The data obtained are based on duplicate measurements for each group, three preparations from each group were stained.

2.5. RNA Isolation, cDNA Synthesis, mRNA Expression, and Primer Design

The RNA from the samples was isolated by using the MagNA Pure Compact RNA isolation kit (Product No: 04802993001, Roche) in the MagNA Pure Compact automated DNA-RNA isolation instrument (Roche Applied Science, Indianapolis, IN, USA). RNA concentration and purity were measured using the NanoDrop spectrophotometer (ThermoFisher Scientific, USA).

Transcriptor First Strand cDNA synthesis kit (Product No: 04896866001, Roche Diagnostics) was used for complementary DNA (cDNA) synthesis. The cDNA synthesis process was carried out according to the experimental protocol included in the kit. The specific primers used in these tests included p53 (F:5′-TCTCCCCAGCAAAAGAAAAA-3′ R:3′-CTTCGGGTAGCTGGAGTGAG-5′), MDM2 (F:5′-CGAGCTTGGCTGCTTCTGGG-3′, R:3′-GCTGGAATCTGTGAGGTGGT-5′) Sin3A (F:5'-TTGTCTCCAATGCTGTTCGC-3', R:3'-GGTTGGCGAATCCTGCGCTC-5') and β actin (ACT) (F:5'-TCCCTGGAGAAGAGCTACG-3', R:3'-GTAGTTTCGTGGATGCCACA-5'). p53, MDM2, and Sin3A gene expressions were analyzed using a LightCycler 480 Real-Time Polymerase Chain Reaction (PCR) detection system (Roche Diagnostics). Reference gene ACT was used in the analysis. The composition of PCR reactions for p53, MDM2, and Sin3A consisted of 5µL cDNA, 8 µL ddH_2O, 5 µL Probe Master mix, 2 µL primer. The program below was selected.

Denaturation : 10 min 95 ^{0}C

Amplification : 10 s 95 ^{0}C, 30 s 60 ^{0}C, 60 s 72 ^{0}C (45 spins)

Cooling : 30 s 40 ^{0}C

The relative amount of mRNA to ACT in each sample was calculated using a relative quantification standard curve.

2.6. Statistical Analysis

Statistical analysis was performed by One-Way ANOVA with Duncan's post hoc test after determining a homogeneous distribution using SPSS software version 20.0 (SPSS Inc. Chicago, IL, USA) and a p value less than 0.05 considered as significant difference between the groups. Linear regression analysis was used to determine the correlation between p53,

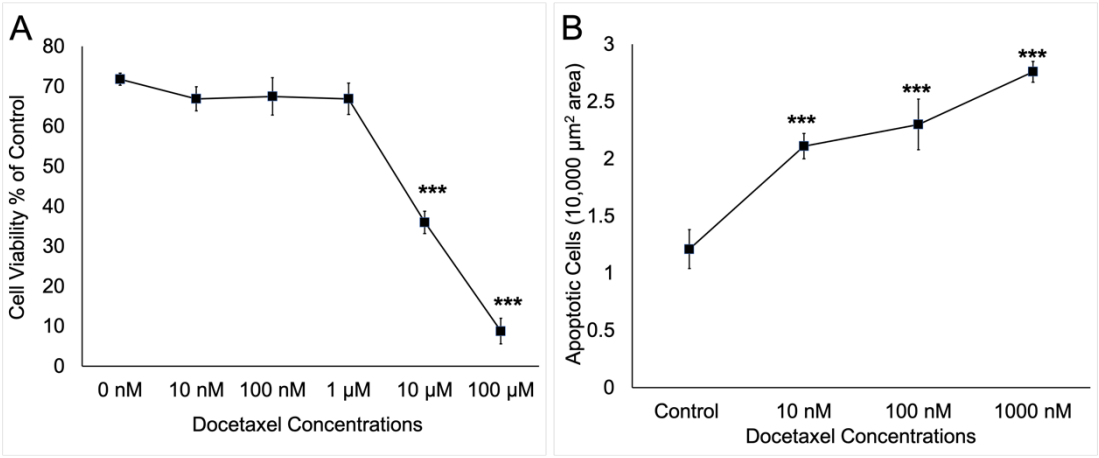
MDM2, and Sin3A variables. Significance between the correlations of dependent and independent variables was determined by means of Fisher's exact test.

3. RESULTS and DISCUSSION

The MCF-7 cell viability in control without any treatment was the highest and the treatments with docetaxel dramatically reduced the cell viability in the 10µM and 100µM concentrations ($p<0.001$). The lowest cell viability rate was in the 100µM docetaxel group and the IC_{50} value for MCF-7 cells was in 10 µM concentration (Figure 1A).

Docetaxel treatments increased apoptotic cell density compared to the control group with an escalating dose manner. The apoptotic cell density was enhanced with the increased concentrations of 10nM, 100nM, and 1µM docetaxel ($p<0.001$) (Figure 1B).

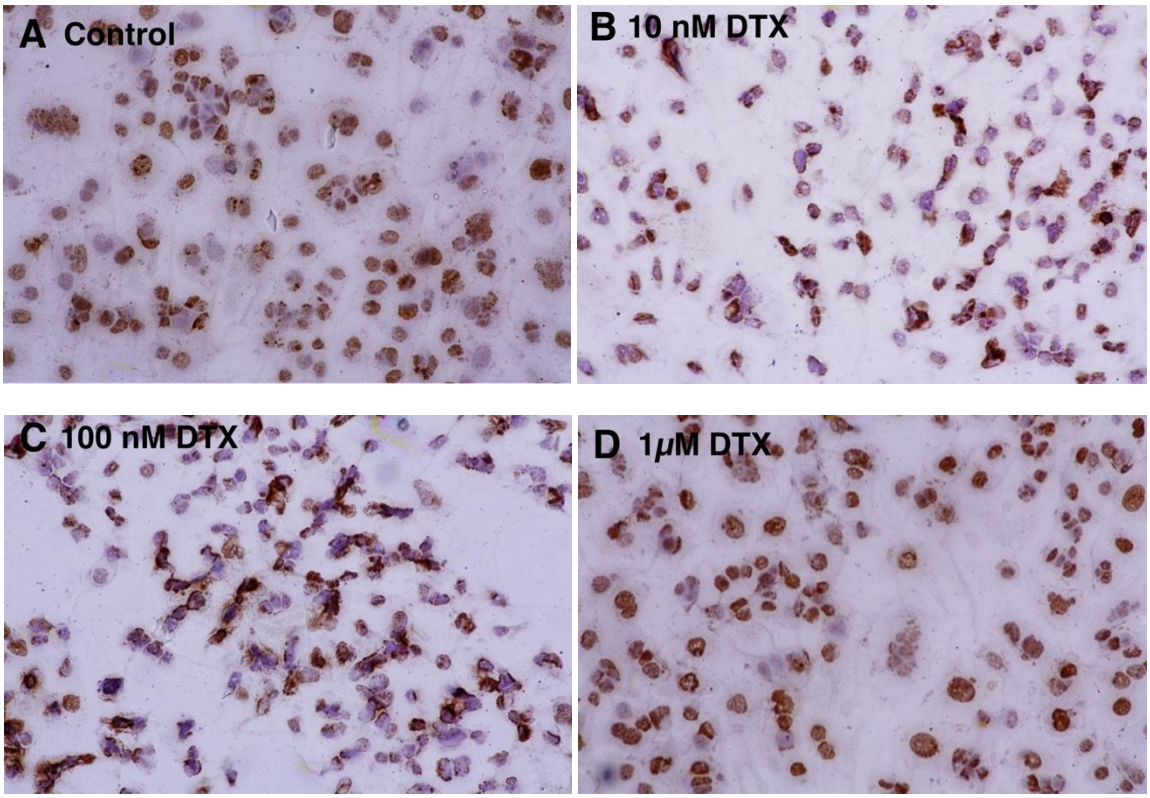
Figure 1. A: Cell viability percentage of MTT measurement in MCF-7 cells treated with docetaxel for 24 h incubation. **B**: Apoptotic cell count of TUNEL assay in MCF-7 cells treated with docetaxel for 24 h incubation. Statistical comparisons were made by One-Way ANOVA with Duncan's post hoc test and a p value less than 0.05 considered as significant difference.



Untreated cells were characterized by a round-shape, and mostly unstained by TUNEL staining. The dark brown cell staining was detected in docetaxel treatment groups indicating apoptosis at an early stage. The percentage of dark brown-stained cells increased in apoptotic MCF-7 cells indicating nuclear DNA fragmentation. The intergroup comparisons of TUNEL staining results of histochemical images are presented in Figure 2.

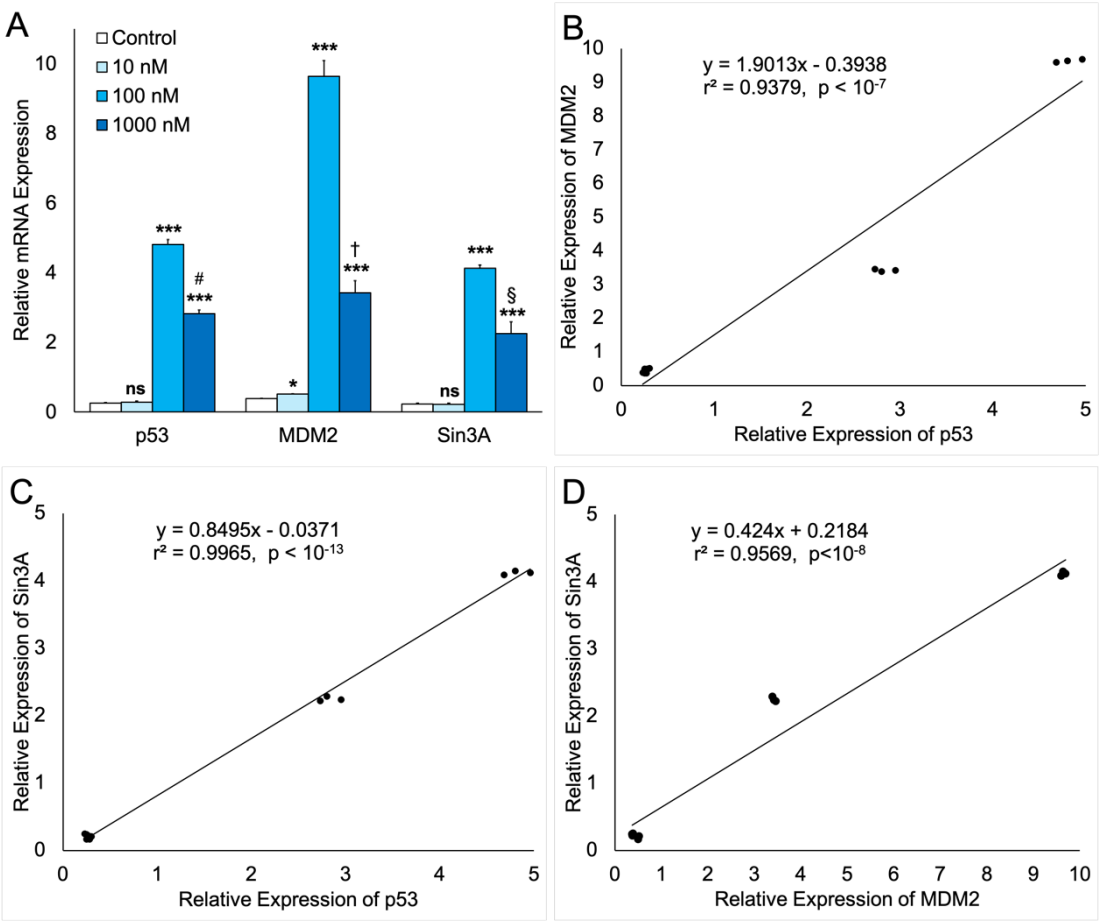
The mRNA expression levels were increased by docetaxel treatment after 24 h incubation. The expressions of p53, MDM2, and Sin3A increased at 100 nM concentration with the same dose-dependent manner compared to control ($p<0.001$) and decreased at 1 µM concentration compared to 100 nM concentration ($p<0.001$). The highest effective level was calculated as 100nM docetaxel concentration ($p<0.001$). Gene expression levels in docetaxel treatment groups are given in Figure 3A. The p53 expression levels were strongly correlated with MDM2 ($r=0.9379$; $p<10^{-7}$) and Sin3A ($r=0.9965$; $p<10^{-13}$) in untreated, 10nM, 100nM and 1µM concentrations. Additionally, the Sin3A expression levels were strongly correlated with MDM2 ($r=0.9569$; $p<10^{-8}$). The p53, MDM2 and Sin3A correlations are given in Figure 3B-D.

Figure 2. TUNEL staining in adhered MCF-7 cells in untreated/Control (**A**), and docetaxel (DTX) concentrations of 10 nM (**B**), 100 nM (**C**) and 1µM (**D**). Brown nucleus indicates TUNEL-positive cells. Untreated cells were characterized by a round-shape, and mostly unstained. The dark brown cells indicate apoptosis at an early stage of nuclear DNA fragmentation by the docetaxel treatment.



In this study, the cell viability decreased and the apoptotic cell density increased after docetaxel treatments with a dose-dependent manner in MCF-7 cells that clearly indicate the chemotherapeutic effectiveness of the drug. The tumor suppressor p53 expression increased at 100 nM docetaxel concentration compared to control after 24 h incubation. However, the p53 inhibitor MDM2 and oncogenic Sin3A expression levels were also increased similar to the p53 level. Apoptotic effect was detected to start at 10 nM and 100 nM docetaxel concentrations based on the TUNEL assay while 10 µM and higher doses were found to be toxic for MCF-7 cells according to MTT analysis. The apoptotic effect of the drug, therefore, seems to depend on gene expressions suggesting p53-mediated apoptosis. These findings reveal that cytotoxicity and apoptosis are mostly related to genetic regulations of chemotherapy in breast cancer.

Figure 3. Relative gene expressions of p53, MDM2 and Sin3A (**A**). The interactions between p53 and MDM2 (**B**), p53 and Sin3A (**C**), MDM2 and Sin3A (**D**).



Statistical comparisons were made by One-Way ANOVA with Duncan's post hoc test and a p value less than 0.05 considered as significant difference. Correlations were determined by linear regression analysis between the variables. Significance between dependent and independent variables was determined by Fisher's exact test.

In previous studies, there has been a higher increase in p53 gene expression with docetaxel administration in the MCF-7 cell line compared to the doxorubicin and resveratrol administration, while there has been a marginal increase in the administration of docetaxel-resveratrol combination (Al-Abd *et al.,* 2011). Additionally, extreme dilutions of paclitaxel and docetaxel alter p53, p21, COX-2, TUBB2A, and TUBB3 gene expressions with little or no cytotoxic/viability effect on MCF-7 cells incubated 72 h (Seker *et al.,* 2018). Similar to these reports, we found that docetaxel administration caused an increase in p53 expression in MCF-7 cells incubated 24 h. The highest p53 expression has been observed in the group treated with 100 nM docetaxel, then the expression levels started to decrease in the group treated with 1 µM docetaxel after 24 h incubation of docetaxel treatment. The decrease in gene expressions at 1 µM concentration is thought to be related to the high concentration of docetaxel due to cytotoxicity.

Most agents restore the function of mutant p53 and inhibit MDM2, which is often overexpressed in human tumors (Vazquez *et al.,* 2008). MDM2, a negative regulator of p53, is an oncogene-functional intracellular protein that is thought to be a significant target for cancer survival. The oncogene MDM2 is an independent negative prognostic marker in breast carcinomas (Turbin *et al.,* 2006) and prevents the apoptosis effect of p53 protein (Freedman *et al.,* 1999). Therefore, blocking the MDM2–p53 interaction can reactive the tumor suppressor gene p53 (Wang *et al.,* 2017; Konopleva *et al.,* 2020). Since MDM2 negatively regulates p53 through the ubiquitin–proteasome pathway, the increase in p53 may also reactivate MDM2

levels to increase because of the feedback mechanism. Docetaxel decreases MDM2 protein level and does not change p53 mRNA level in LNCaP cells (bearing wild-type p53) for human prostate adenocarcinoma (Gan *et al.,* 2011). In advanced-stage breast cancer, docetaxel decreases MDM2 protein level compared to the combination of methotrexate and 5-Fluorouracil (Sjostrom *et al.,* 2000). In this study, the increase in MDM2 gene expression has been observed in the 10 and 100 nM docetaxel concentrations compared to the control group, and the highest expression has been in the 100 nM docetaxel concentration which is similar to the p53 expression reaction. MDM2 level decreased at 1 µM docetaxel as seen in p53 level. We found that an increase and a decrease in p53 level that is strongly correlated with MDM2 level in MCF-7 cells treated with docetaxel. The decrease in p53 gene expression may be due to the decrease in the MDM2 protein level. Although the p53 level may indicate the chemotherapeutic effectiveness of a drug, cancer variability and drug treatment affect the genetic response of the cells.

We found an increase in Sin3A gene expression in the MCF-7 cell line with docetaxel administration. However, there is a limited report investigating the effect of docetaxel on Sin3A in the previous studies. Sin3A and Sin3B differentially regulate breast cancer metastasis (Lewis, 2016). Low levels of Sin3A and Sin3B expressions have been associated with the progression of many cancers. Ellison-Zelski *et al*. (2010) examined the function of Sin3A in the development and gene expression of breast cancer cells. They observed that the absence of Sin3A inhibits the growth of breast cancer cells with an increase in apoptosis. In the evaluation of both ERα-positive and ERα-negative cell lines, the effects of Sin3A on growth are found to be specific to cell type, and Sin3A expression provides maximum growth only in ERα-positive cells suggesting that the Sin3A protein is significantly increased by estrogen. When the Sin3A gene is transferred to MCF-7 cells, Sin3A suppresses the expression of key proapoptotic genes. Sin3A is important in the regulation of development, survival, and gene expression in ERα-positive breast cancer cells that Sin3A could be a new therapeutic target. Furthermore, Yang *et al*. (2018) suggested that LSD1/Sin3A/HDAC complex could be a target for breast cancer therapeutic strategies. Additionally, the interaction between p53 and Sin3A has been studied in MCF-7 and A2780 ovarian cancer cells with a DNA damaging agent. The Sin3A-p53 complex increased approximately four times in MCF-7 cells (Murphy *et al.,* 1999). The mSin3A corepressor binds to both wild-type and mutant p53 accompanied by DNA binding. Zilfou *et al*. (2001) suggested that Sin3A stabilizes p53 and inhibits the degradation of p53 by MDM2 in MCF-7 cells. In this study, Sin3A gene expression levels were high at the 100 nM and 1 µM docetaxel concentrations compared to the control but the level decreased at 1 µM compared to 100 nM concentration. The reduction in Sin3A mRNA has been reported to be correlated with the recurrence of ER-positive breast cancers since the Sin3A mutation has lost its transcriptional repression function due to its cytoplasmic localization (Watanabe *et al.,* 2018). The Sin3A mutation enhances MCF-7 cell proliferation. The increase in p53 expression strongly correlated with the increase in Sin3A at the same doses, herein. ER-positive MCF-7 breast cancer cells are prone to Sin3A elevation and this increase may be related to cell proliferation.

4. CONCLUSION

The efficacy of docetaxel is supported by p53 overexpression according to the enhancement of apoptotic cell density. Sin3A expression may lose its transcriptional repression function related to the drug concentration. The response of MCF-7 cells to docetaxel treatment seems to be in favor of p53 gene overexpression. In conclusion, MCF-7 breast cancer cells respond to docetaxel treatment in a dose-dependent manner and may thus further regulate the interaction of tumor suppressor p53 expression with MDM2 and Sin3A. Furthermore, docetaxel protects p53 from MDM2-mediated degradation and inhibition of Sin3A-mediated cell proliferation according to the enhancement of apoptotic cell density and the reduction of cell

viability. The present study may lead the evaluation of the treatment of breast cancer regarding the genetic and chemotherapeutic factors that have a regulatory role for p53.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Nezahat Kurt: Conceptualization, Writing- Original draft preparation, Writing – review, Editing. **Nuri Bakan**: Supervision. **Adem Kara:** Methodology, Data collecting. **Seckin Ozkanlar:** Methodology, Data collecting. **Eda Balkan**: Methodology, Data analysis. **Fatma Betul Ozgeris:** Methodology, Data analysis.

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Determination of Different Biological Activities of Methanolic Extracts of Fruits of *Passiflora ligularis* Juss. and *Passiflora edulis* Sims.

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Abstract

Antioxidants are important for the treatment of various diseases, including cancer that causes human death. This study was conducted to determine the antioxidant activities of *Passiflora edulis* Sims. and *Passiflora ligularis* Juss. fruit methanol extracts (DPPH, ABTS, β-carotene, FRAP and CUPRAC), total secondary metabolite amount (Phenolic, Flavonoid, Tannin), as well as to determine the anticancer potential against human colorectal cancer (HT-29) and human lung cancer (A549). Results indicated that the *P. ligularis* fruit methanol extracts exhibit high biological activity in all antioxidant activity experiments (DPPH, 59.30±1.28%, ABTS, 57.60±0.55%, β-carotene, 88.17±1.86%, FRAP, 1.07±0, 6 mg TE/g, CUPRAC, 9.01±0.38 mg TE/g). There is a good correlation between antioxidant activity and total phenolic. At 8 mg/mL, the extract of *P. ligularis* showed higher cytotoxicity against A549 cells than *P. edulis* extract. At 2 mg/mL and lower concentrations, the viability of HT-29 cells treated with both extracts was above 50%. In conclusion, *P. ligularis* and *P. edulis* are potential sources for future studies in the pharmaceutical industry.

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1. INTRODUCTION

Turkey has a rich floristic structure as it hosts plant species with different morphological and anatomical characteristics. The origin of this diversity comes from Turkey's unique location as a neighbor of Europe, Asia, and Africa continents, in addition to its habitat diversity, altitude differences, climate diversity. Our country is one of the richest countries in Europe with its genetic, species, and ecosystem diversity and its unique endemic species (Şekercioğlu *et al*., 2011; Gross, 2012). It is known that there are more than 3000 endemic species and approximately 12000 plant taxa in Turkey (Özcan, 2021). In this context, the treatment of diseases is carried out with the use of natural plant extracts, animals, and various microorganisms. With their extensive chemical diversity, plants have been used in traditional medicine since ancient times and have become widespread all over the world, becoming the first source of natural medicine and edible food products used by people (Wachtel-Galor and Benzie, 2011). Herbal medicines are used in many areas such as eliminating global health problems, reducing the negative effects of events such as stress and aging, and in the food sector (Ekor, 2014). In addition to the lack of information on issues such as the uncertainty of dosage and the toxicity of some species in the use of plants in traditional medicine, more research is needed to determine their possible biological activities (Plančić *et al*., 2014). Plants are raw materials in high demand in the food and health industry due to the secondary metabolites they

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produce. Meteorological conditions, geographical location, and growing conditions affect secondary metabolite production (Güven and Gürsul, 2014; Topçu and Çölgeçen, 2015). Secondary metabolites are divided into three main groups: terpenes and terpenoids, alkaloids, and polyphenolic. These metabolites have different biological activities such as antioxidant, anticancer, antihelminth, larvicidal, anti-inflammatory, antimicrobial, anti-mutagenic, and anti-tumor (Aydın *et al*., 2014). Antioxidants are substances that are easily absorbed by the body, have a low concentration compared to potentially oxidizable substrates, and can reduce the oxidation of this substrate. Antioxidants are substances that interfere with the oxidation process by stopping the formation of free radicals and chelating redox metals and play a role in the prevention of diseases such as cancer, heart diseases, cataracts, and aging (Poljsak *et al*., 2013). Antioxidants obtained from natural products in drug production have fewer side effects than synthetic antioxidants. Therefore, the interest in the search for antioxidant substances from natural products has gained importance over time (Abdioğlu, 2019). Stress conditions such as drought, temperature differences, and radiation that may adversely affect the plant create various reactive oxygen species (ROS). Examples of reactive oxygen species are nitrogen, free radical species, and sulfur derivatives. Species included in ROS contain one or more unpaired electrons and cause high reactivity (Dumanović *et al*., 2021). Cancer is responsible for the death of many people in the world. Chemotherapy routinely used for cancer treatment has various side effects such as drug resistance and undesirable toxicity. Plants still maintain their potential as new sources of drugs against cancer (Desai *et al*., 2014). *Passiflora* L., which has more than 500 species in the Passifloraceae family, is often called passion fruit. The most well-known and widely used species is *Passiflora edulis* Sims. It is used as a sedative in America and European countries (Silva *et al*., 2013). *P. edulis* is used in many pharmaceutical preparations and in the food industry (Petry *et al*., 2001). The leaves of the *Passiflora ligularis* Juss. plant, which is native to the Americas, has been used in traditional medicine for the treatment of mumps, removing bruises, controlling infections, relieving low back pain and digestive system disorders (Echeverri *et al*., 2021). This study, it was aimed to determine the potential antioxidant activity (DPPH, ABTS, CUPRAC, FRAP and β-carotene test) and total secondary metabolite (Phenolic, Flavonoid and Tannin) amounts of *P. edulis* and *P. ligularis* fruit methanol extracts. The present study also aimed to evaluate the effects of methanolic extracts of fruits of both *P. ligularis* and *P. edulis* on the viability of human colorectal cancer (HT-29) and human lung cancer (A549) cells.

2. MATERIAL and METHODS

2.1. Chemical

DPPH, ABTS, BHA, β-carotene, trolox, FCR, Sodium carbonate, gallic acid and quercetin were obtained from Sigma-Aldrich (USA).

2.2. Plant Materials and Extract Preparation

The fruits of *P. edulis* and *P. ligularis* were collected from the land of the Azerbaijan National Academy of Sciences, Dendrology Institute. The collected plant fruits were dried and grind into pieces. Then, small pieces of dried fruits were soaked in methanol (1:10). It was left at 55 °C for 6 hours with a shaker incubator (Thermo Scientific MaxQ 4450). The mixture was filtered using filter paper. Then, the solvent was removed from the filtrate by rotary evaporator (Heindolph LABOROTA 4011) and it was kept in a lyophilizer (Thermo Savant Modulyo D) for 8 hours to remove the water inside. The extracts obtained were stored at -20 °C until they were used in experiments.

2.3. Antioxidant Activity

2.3.1. DPPH Radical Scavenging Assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity experiment was carried out using the method of Turan and Mammadov (2018). 4 mL of DPPH solution was added to 1 mL of BHA (for positive control) and extract solutions were prepared at different concentrations (1.0, 1.2, 1.4, 1.6 mg/mL). As a negative control, 4 mL of DPPH solution was added to 1 mL of solvent. It was measured at 517 nm after 30 minutes of incubation in the dark at room temperature. The percent antioxidant activity value (AA%) was found according to the following formula (1):

$$AA\%=[(A_c-A_s)/A_c] \times 100 \quad (1)$$

A_c; Absorbance of the control, A_s; Absorbance of the extract or standard

2.3.2. ABTS Radical Scavenging Assay

ABTS (2,2-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging activity Re *et al*. (1999) using the method specified. 4.5 mL ABTS solution was added on top of 0.5 mL extract or standard solutions (BHA) prepared at five different concentrations (0.05-0.25 mg/mL). As a negative control, 4.5 mL of ABTS solution was added to 0.5 mL of solvent. It was measured at 734 nm after incubation for 30 minutes at room temperature. The percent antioxidant activity value (AA%) was found according to the following formula (2):

$$AA\%=[(A_c-A_s)/A_c] \times 100 \quad (2)$$

A_c; Absorbance of the control, A_s; Absorbance of the extract or standard

2.3.3. β-Carotene/Linoleic Acid Method

The method developed by Amarowicz *et al*. (2004) was used in the β-carotene/linoleic acid assay. 24 mL of β-carotene solution was added to 1 mL of extract or BHA (for positive control). Initially, the initial absorbance of the mixtures was measured at 470 nm. Afterwards, it was incubated at 50 °C for 2 hours and measurements were made every half hour. As negative control, 24 mL of β-carotene solution was added to 1 mL of solvent. Total antioxidant activity was calculated using the following formula (3):

$$AA\% = 1-[(A_o(0)-A_c(0))/(A_o(d)-A_c(120)) +(A_s(0)-A_s(120)))] \times 100 \quad (3)$$

$A_o(0)$; The absorbance of the extract at 0 min, $A_c(0)$; The absorbance of the control of the extract at 0 min, $A_o(120)$; Absorbance of the extract at 30., 60., 90. or 120. minutes, $A_c(d)$; The absorbance of the control of the extract at 30, 60, 90 or 120 minutes, $A_s(0)$; The absorbance of the positive control at 0 min, $A_s(120)$; It represents the absorbance of the positive control at 120 minutes.

2.3.4. FRAP (Iron Reducing Antioxidant Power) Method

The method made by Benzie and Strain (1996) was used in the iron reducing antioxidant power (FRAP) experiment. To determine the FRAP, 2 mL of FRAP solution was added onto the 1 mg/mL extract solution. It was then measured at 595 nm after standing at room temperature for 30 minutes. Trolox (0.05-0.2 mg/mL) was used as standard. The calibration graph was obtained using the standard.

2.3.5. CUPRAC (Copper Reducing Antioxidant Power) Method

The copper reducing antioxidant power capacity (CUPRAC) was performed according to the method described by Apak *et al*. (2004). To determine the CUPRAC, 3 mL of CUPRAC solution was added to 0.5 mL of extract solution (1 mg/mL) and then incubated for 30 minutes at room temperature and measured at 450 nm. Trolox (0.01-0.05 mg/mL) was used as standard. The calibration graph was obtained using the standard.

2.4. Quantification Analysis of Extracts

2.4.1. Determination of Total Phenolic Quantity

Total phenolic quantification was carried out according to the method performed by Singleton and Rossi (1965). To determine the phenolic content, 1 mL of Folin-Ciocalteu reagent (FCR) was added onto the 1 mg/mL extract solution. After adding 46 mL of distilled water, it was left for 3 minutes. At the end of 3 minutes, 3 mL of sodium carbonate was added and left at room temperature for 2 hours. Measured at 760 nm after 2 hours. Gallic acid (0.01-0.05 mg/mL) was used as a standard. The calibration graph was obtained using the standard.

2.4.2. Determination of Total Flavonoid Quantity

Determination of total flavonoid substance was conducted to the method of Aryal *et al*. (2019) with some modifications. To determine the amount of flavonoids, 0.2 mL of sodium acetate was added onto the 1 mg/mL extract solution. Then, 1 mL of aluminum chloride and 5.6 mL of distilled water were added. It was measured at 415 nm after 30 minutes at room temperature. Quercetin (0.01-0.05 mg/mL) was used as a standard. The calibration graph was obtained using the standard.

2.4.3. Determination of Total Tannin Quantity

Bekir *et al*. (2013) method was used for determining the content of total tannin.0.5 mL of extract or 1.5 mL of vanillin solution was added to the standard of different concentrations in test tubes in an ice-filled container. After the vanillin solution was added, it was incubated at room temperature for 15 minutes. After 15 minutes, its absorbance was measured at 500 nm. For the calibration graph, the same procedures will be performed for catechin solutions at different concentrations (0.01 mg/mL, 0.02 mg/mL, 0.03 mg/mL, 0.04 mg/mL, 0.05 mg/mL).

2.5. Cell Culture and MTT Assay

A549 (human lung cancer cell line) and HT-29 (human colorectal cancer cell line) cells were used in the present study. The cells were grown in RPMI-1640 medium (Diagnovum) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Diagnovum) and penicillin and streptomycin (100 U/mL-100 µg/mL, Diagnovum) solution, and cultured at 37°C in 5% CO_2.

MTT assay (Mosmann, 1983) was used to determine the effect of methanolic extracts of fruits of both *P. ligularis* and *P. edulis* on cell viability. The cells were treated with serial dilutions of the extract for 72 h. Briefly, the cells were seeded into 96-well microplates ($1x10^4$ cells/well) and incubated for 24 h. Then, each concentration of the extracts was added to the wells in question. Untreated cells served as a control group. After 72 h incubation, the medium in each well was replaced with 100 µL of fresh medium and 10 µL of MTT (5 mg/mL) was added to the wells. Following to the incubation of the microplates for 4 h, 100 µL of DMSO was added to each well and microplates were shaken for 6 minutes at 150 rpm. The absorbances were read at 540 nm using a microplate reader (Thermo Scientific, Multiscan GO). The percentage of cell viability was calculated using these absorbance values of treated and untreated cells.

2.6. Data Analysis

In all experiments, each concentration was performed in three replicates and the results were presented as the mean ± standard error.

3. RESULTS and DISCUSSION

3.1. Antioxidant Activity

In this study, five different methods were used to determine the antioxidant activities of methanol extracts obtained from *P. edulis* and *P. ligularis* fruits, and the results are given in Table 1. *P. ligularis* fruit extract (59.30 ± 1.28 %) showed the highest activity in terms of DPPH scavenging activity. Both extracts were observed to exhibit a lower activity in comparison to the positive control BHA in terms of DPPH and ABTS assays. DPPH radical scavenging activity was determined as 27.93 ± 2.04 % and 59.30 ± 1.28 % for *P. edulis* and *P. ligularis* extract, respectively. ABTS radical scavenging capacity of *P. ligularis* extract (57.60 ± 0.55 %) is higher than *P. edulis* extract (17.77 ± 0.84 %).

Table 1. Antioxidant activity of *P. edulis* and *P. ligularis* extracts.

Plant	DPPH (%)	ABTS (%)	β-Carotene/Linoleic Acid (%)	FRAP (mg/mL)	CUPRAC (mg/mL)
P. edulis	27.93 ± 2.04	17.77 ± 0.84	71.05 ± 3.65	0.67 ± 0.02	2.47 ± 0.07
P. ligularis	59.30 ± 1.28	57.60 ± 0.55	88.17 ± 1.86	1.07 ± 0.6	9.01 ± 0.38
BHA	93.26 ± 0.89	88.65 ± 0.42	91.15 ± 0.24		

The effects of *P. ligularis* and *P. edulis* fruit extracts on β-carotene bleaching are 88.17 ± 1.86 % and 71.05 ± 3.65 %, respectively. According to this assay, both extracts showed a value close to the positive control. The copper (II) ion reducing power activity of *P. ligularis* extract at 1 mg/mL was measured as 9.01 ± 0.38 mg TE/g and it was a higher value than that of *P. edulis* extract. In the FRAP assay, *P. ligularis* extract at 1 mg/mL was showed higher antioxidant activity than *P. edulis* extract. In all antioxidant activity experiments conducted in this study, it was determined that *P. ligularis* methanol fruit extract showed higher activity than *P. edulis* methanol fruit extract. It is thought that the reason for this may be related to the phenolic compounds in the *P. ligularis*.

There are studies to determine the potential antioxidant activities of extracts obtained from different parts of *P. edulis* and *P. ligularis* species with different solvents. Silva et al. (2013) reported that water extract obtained from *P. edulis* leaves (1100 µg/mL, IC_{50}) could be a potential source of antioxidants. In another study, the ethanol extract obtained from the peel of the fruit of *P. edulis* species was determined to show a lower antioxidant activity compared to the positive control (Wong *et al*., 2014). Sunitha and Devaki (2009) reported that the ethanol extracts of *P. edulis* leaves showed high antioxidant activity (58.17 ± 2.45 %). On the other hand, in our study, it was observed that *P. edulis* methanol fruit extract exhibited a lower antioxidant activity. It is thought that the differences between the studies and our results are probably due to the use of different solvents and other parts of the plant. Saravanan and Parimelazhagan (2014) investigated the potential antioxidant activities of extracts of *P. ligularis* fruit prepared with different solvents and revealed that the acetone extract (19.13 µg/mL, IC_{50}) of its fruit showed high antioxidant activity. Compared to our results, data obtained from the extract of *P. ligularis* fruit prepared with different solvents show that the methanol fruit extract of *P. ligularis* in the present study has a lower activity than their acetone extract. These differences may be due to the solvent used.

3.2. Quantification Analysis of Extracts

The results of total secondary metabolite amounts of *P. ligularis* and *P. edulis* fruit methanol extracts are shown in Table 2. When the phenolic content of both extracts was compared, it was observed that the *P. ligularis* extract was higher. The total phenolic content of *P. ligularis* and *P. edulis* extracts were found to be as 3.37 ± 0.11 mg GAE/g and 1.44 ± 0.26 mg GAE/g, respectively.

Table 2. Secondary metabolite amounts of *P. edulis* and *P. ligularis*.

Plant	Phenolic (mg GAE/g)	Flavonoid (mg QE/g)	Tannin (mg CE/g)
P. edulis	1.44 ± 0,26	1.23 ± 0.24	0.22 ± 0.13
P. ligularis	3.37 ± 0.18	0.65 ± 0.07	0.24 ± 0.12

Total flavonoid amount of *P. edulis* is higher than that of *P. ligularis* unlike their phenolic content. The total amount of flavonoid of *P. edulis* and *P. ligularis* extract was determined to be as 1.23 ± 0.24 mg QE/g and 0.65 ± 0.07 mg QE/g, respectively. the total tannin content was the highest in the *P. ligularis* extract (0.24 ± 0.12 mg CE/g).

Saravanan and Parimelazhagan (2014) revealed that total phenolic, flavonoid, and tannin contents of the acetone extract were 640.70 mg GAE/g, 387.33 mg RE/g and 214.30 mg GAE/g, respectively. Wong *et al*. (2014) reported that the total phenolic content of aqueous ethanol extract obtained from the bark of *P. edulis* and was 15.84 ± 0.63 µg GAE/g extract. Ramaiya *et al*. (2014) investigated the total phenolic contents of extracts obtained from different parts of *Passiflora quadrangularis* L., *P. maliformis* L. and *P. edulis* with different solvents. The phenolic contents for the leaves and stems extracts were ranged from 3.32 to 1.23 g GAE/100 g and 3.74 to 1.03 g GAE/100 g, respectively. In another study, total phenolic contents were detected to be higher in *P. alata* Curtis extract (171 ± 1.6 µg/mg extract) than *P. edulis* (92.5 ± 2.2 µg/mg extract) extract (Rudnicki *et al*., 2007). As a result, *P. edulis* and *P. ligularis* extracts contain lower amounts of secondary metabolites according to the studies aforementioned. According to the results different solvents used, the plant part examined and the plant region was chosen maybe affect the bioactivity of plants. In our study, there is a positive correlation between total phenolic and antioxidant activity.

3.3. Cell Culture and MTT Assay

As shown in Figure 1, the viability of A549 cells was determined to be above 50% when the cells were treated with the extract of *P. edulis* at 4 mg/mL and lower concentrations or the extract of *P. ligularis* at 1 mg/mL and lower concentrations. At 8 mg/mL, the extract of *P. ligularis* was more effective on the viability of A549 cells than *P. edulis* extract.

It was observed that the greatest decrease in HT-29 cell viability was due to the *P. ligularis* extract at 8 mg/mL, as in A549 cells. At 2 mg/mL and lower concentrations, the viability of HT-29 cells treated with both extracts separately was above 50%. The future studies on the evaluation of the cytotoxic potential of the extracts used here will provide a contribution to the literature.

Figure 1. *P. edulis* methanol **(**PEMM) and *P. ligularis* methanol (PLM) extracts on viability of A549 cells

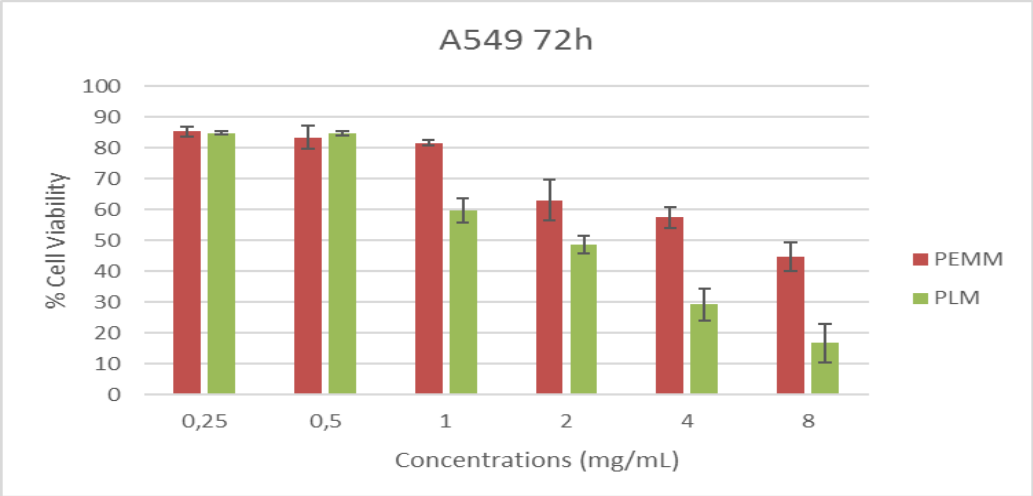
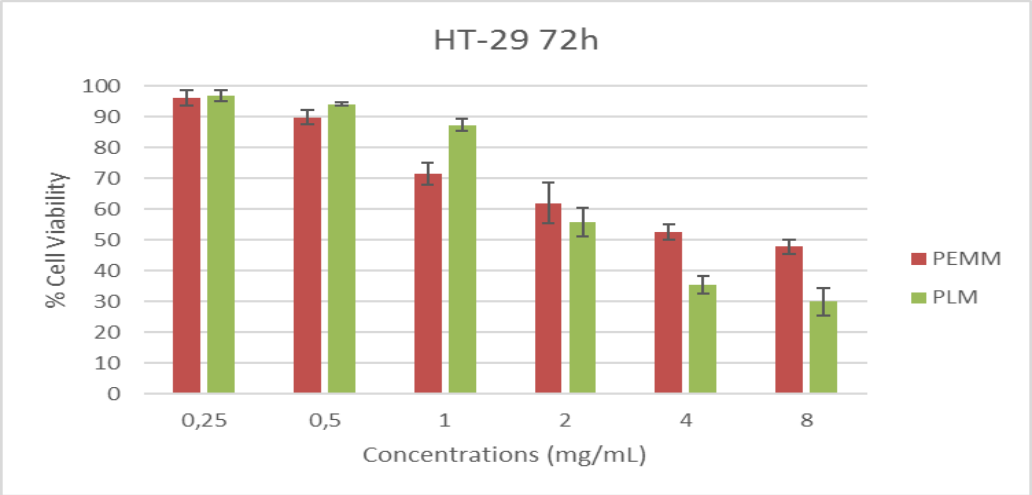


Figure 2. *P. edulis* methanol **(**PEMM) and *P. ligularis* methanol (PLM) extracts on viability of A549 cells



The cytotoxic potential of different *P. edulis* on different cancer cells was assessed in the previous studies. Unlike our study, the ethanolic extract of leaves of *P. edulis* was reported to decrease in viability of the colon cancer cell lines SW480 and Caco2 (Ramírez *et al*., 2017). Aguillón *et al*. (2018) displayed the cytotoxicity of juice and leaf extract of *P. edulis* on HepG2 (liver cancer) cells. Mota *et al*. (2018) evaluated the antitumor activity of the crude extract (HCE) and the supercritical fluid extract with ethanol as co-solvent (SFEtOH) from *P. edulis* against MCF-7 (human breast adenocarcinoma) cell lines, and reported that SFEtOH showed higher antitumor activity than HCE. To our knowledge, the present study the first time evaluated the cytotoxicity of the methanolic extracts of fruits of both *P. ligularis* and *P. edulis* on the viability of A549 and HT-29 cells. The differences in the cytotoxicity of the extracts may be caused by the cells tested.

4. CONCLUSION

In this study, the effects of *P. edulis* and *P. ligularis* methanol extracts on antioxidant activity, total substance content and viability of different cell lines were investigated. There are no studies on the viability of A549 and HT-29 cells of *P. edulis* and *P. ligularis* methanol extract in the literature. In this study, the biological activities of both species were revealed. It has been shown that there may be a significant linear correlation between the phenolic content of the extracts and their antioxidant activity. Further research is required to determine the potential health benefits of in vivo and elucidate their mechanism of action.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Vusala Badalova: Writing, Editing, Validation. **Mehmet Ozgur Atay:** Laboratory work. **Buse Ardil:** Experiment design, Supervision, Statistical analysis, Validation.

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Biosynthetic Capacity of *Artemisia annua* L. "Hairy" Roots

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Abstract

Transformation using *Agrobacterium rhizogenes* is a widely used method for obtaining "hairy" roots of different plant species. Such roots are characterized by rapid growth under simple growing conditions. Incorporation of bacterial *rol* genes into the plant genome after the transformation can lead to changes in bioactive compounds synthesis in "hairy" roots. Analysis of the content of carbohydrates and amino acids in *Artemisia annua* L. "hairy" roots was the aim of the work Inulin content in transgenic root lines increased by 1.52 - 1.86 folds compared to the roots of the control plants. The "hairy" roots lines, in contrast, to the control plants, did not contain inositol. There was a decreased glutamine (up to 5.92 folds) and increased arginine hydrochloride (up to 1.72 folds) and proline (up to 2.53 folds) content in transformed root lines. Seven of the total identified amino acids are indispensable amino acids: valine, isoleucine, leucine, lysine, threonine, tryptophan, and phenylalanine. The results of the study demonstrated that the genetic transformation of *A. annua* plants has led to changes in the accumulation of carbohydrates and amino acids (both quantitatively and qualitatively). "Hairy" root lines with increased content of individual carbohydrates and amino acids were identified.

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1. INTRODUCTION

Artemisia annua L. (annual wormwood) is a medicinal plant traditionally used for malaria treatment (Daddy *et al*., 2017). Artemisinin, which is present in extracts of wormwood, has been identified as an active component of this plant, which causes antimalarial properties (de Vries and Dien, 1996; Brown, 2010; Haynes, 2006). However, wormwood plants contain other biologically active compounds (Mirbehbahani *et al*., 2020). In recent years, drugs with *A. annua* extracts have attracted attention because of their antitumor properties (Mashati *et al*., 2019; Rassias and Weathers, 2019). Extracts of this plant also have anti-inflammatory, antibacterial, and antimicrobial properties. There is information about the activity of wormwood against SARS-CoV-2 (Nair *et al*., 2021). *A. annua* extracts are also considered promising for the creation of wound healing agents. In particular, such compounds are not cytotoxic and at the same time exhibit antibacterial activity (Mirbehbahani *et al*., 2020).

It is known that plants of different species of the *Artemisia* genus, used in traditional medicine, contain water-soluble polysaccharides with a wide range of biological properties (Corrêa-Ferreira *et al*., 2014). The water-soluble carbohydrate fraction isolated from *A. iwayomogi* Kitam. modulated the functional differentiation of bone marrow dendritic cells (Lee *et al*., 2008) and showed immunomodulatory and antitumor activity in mice (Koo *et al*., 1994).

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Fractions of polysaccharides from *A. tripartita* Rydb. leaves showed immunomodulatory and antiradical activity (Xie *et al*., 2008). Fructan inulin was studied as the main polysaccharide in *A. vulgaris* L. (Corrêa-Ferreira *et al*., 2014).

To obtain naturally synthesized biologically active compounds, plants specially grown in cultivation fields or those grown and harvested in natural conditions are currently used. Such plant raw materials are carefully analyzed for purity and the presence of toxic compounds. However, due to air, soil, and water pollution, the presence of toxic compounds in such raw materials may occur. Such pollution leads to significant rejection of the material for the extraction of valuable compounds. Significant dependence of the synthesis and accumulation of biologically active compounds on climatic conditions (temperature, precipitation, soil composition, etc.) can be named as the disadvantage of using collected natural raw materials for the obtaining of plant-derived bioactive compounds for medical purposes (Aires *et al*., 2011; Pék *et al*., 2013). In addition, the collection of plants is possible only during the limited growing season. At the same time, modern possibilities of genetic engineering allow to create of "artificial" producers of biologically active substances. Such producers include "hairy" root cultures obtained by plant transformation using phytopathogenic bacteria *Agrobacterium rhizogenes* (Srivastava and Srivastava, 2007). Such roots are characterized by the rapid growth of biomass under simple growing conditions, they do not require additional lighting and the use of expensive reagents (Uozumi, 2004; Srivastava and Srivastava, 2007; Cardillo *et al*., 2016). In addition, since genetic transformation and transfer of *rol* genes to the plant genome can lead to activation of the synthesis of bioactive compounds in the "hairy" roots, selection using available methods allows obtaining lines that behave as superproducers of valuable compounds. A number of studies have identified the possibility of increasing the production of polyphenols, essential oils, and other substances in cultures of "hairy" roots of different plant species. For example, the roots of *Raphanus sativus* L., obtained by transformation using *A. rhizogenes* MTCC 2364 and MTCC 532, synthesized quercetin in an amount of up to 114.8 mg / g, which was higher than in the control (Balasubramanian *et al*., 2018).

The change in the level of synthesis of secondary metabolites in the transgenic roots of *Fagopyrum tataricum* (L.) Gaertn. was revealed (Mi *et al.,*2020). Our experiments showed an increase of flavonoids content and an increase of antioxidant activity in the "hairy" roots of *Artemisia vulgaris* (Matvieieva *et al*., 2019). The sucrose content in the transgenic roots of *A. vulgaris* was higher than in the control (Drobot *et al*., 2017). The fructose content in the "hairy" roots of *A. dracunculus* L. increased compared to the untransformed control (Drobot *et al*., 2017). The concentrations of amino acids such as lysine, isoleucine, valine, histidine, and phenylalanine were higher in the "hairy" roots of *Fagopyrum esculentum* Moench than in the control (Gabr *et al*., 2019). In these roots, the increased level of flavonoid accumulation correlated with high antioxidant activity. In addition, the transgenic roots differed from the control not only in the total amount of metabolites but also in the spectrum of detected flavonoids. In particular, the authors in that study detected hesperidin and kaempferol-3-rutinoside, absent in the control roots.

The use of wormwood "hairy" root culture as a new type of medicinal biotechnological raw material requires a comprehensive study of other metabolites such as amino acids and carbohydrates, along with the evaluation of the content of basic pharmaceutically active compounds such as artemisinin and flavonoids. During extraction with hot water, carbohydrates that have certain therapeutic properties can also be extracted. Thus, the results of studies of the amino acid composition of *A. heptapotamica* Poljakov and *A. albida* Willd. ex Ledeb. have been published recently (Mukatay *et al*., 2021). In these plants a significant content of aspartic and glutamic acids, alanine, and proline was found. The authors (Ochkur *et al*., 2013) also studied the amino acid content in *Artemisia* spp. plants. Valine, isoleucine, phenylalanine,

leucine in amounts up to 102 mg / 100 g dry weight were found in *A. argyi* H. Lév. & Vaniot (Kim *et al*., 2015).

In our work, we used NMR to analyze aqueous (D_2O) extracts of "hairy" roots of *A. annua* L. The aim of this study was to compare the content of carbohydrates and amino acids in the "hairy" root lines, which were obtained by transformation of *A. annua* plants using *A. rhizogenes*, and in the roots of the control plants cultivated in the same *in vitro* conditions.

2. MATERIAL and METHODS

2.1. Plant Material

We used obtained by us earlier (Drobot *et al*., 2016) "hairy" roots of *A. annua* which were grown for 4 weeks on solidified Murashige and Skoog (1962) nutrient medium with half reduced macrosalts content and 20 g/L of sucrose at a temperature of +24° C. The control plants were cultivated at the same *in vitro* conditions. The "hairy" roots and control plants were collected from the medium, washed with distilled water, frozen at -70 °C and lyophilized by Labconco Freeze Drier 4.5.

2.2. Extract Preparation and NMR Analysis

To prepare extracts, 0.07 g of each sample (pre-crushed lyophilized roots) was placed into 10 mL glass containers and mixed with 2 mL of deuterated water (D_2O, degree of deuteration - 99.8%), containing tert-butanol as a reference compound in a concentration of 0.05%. Samples in closed containers were kept at room temperature with periodic stirring for 24 hours. Then the extracts were filtered and transferred into ampoules for the subsequent recording of NMR spectra. Spectra on ^{1}H and ^{13}C were recorded on an AVANCE-500 NMR spectrometer (Bruker, Germany) with operating frequencies of 500 MHz and 125 MHz for ^{1}H and ^{13}C nuclei, respectively, at a temperature of 293° K. ^{1}H spectra were recorded in "quantitative" mode. The number of accumulations for proton spectra was 512, for carbon - not less than 15000 with a relaxation delay of 3 sec. Chemical shifts were determined using added t-BuOH, for the methyl groups of which in ^{1}H spectra δ = 1.24 ppm and in the ^{13}C spectra = 30.29 ppm. Spectral data of individual carbohydrates and amino acids were obtained by us earlier (Skakovski *et al*., 2013; Shysh *et al*., 2017). Besides this, available data from the site https://www.chemicalbook.com were used to identify compounds in the spectra of the studied extracts. Quantitative measurements were performed based on a comparison of the integrated intensities of the proton lines of the analyzed compounds with the integrated intensity of methyl protons of added t-BuOH in known concentrations. The relative integration error was 5%.

3. RESULTS and DISCUSSION

From the analysis of spectra, it was found that the extracts contained six carbohydrates: galactose, glucose, inositol, inulin, sucrose, and fructose. Some amino acids, in particular, γ-aminobutyric, alanine, arginine, asparagine, valine, glutamine, isoleucine, leucine, lysine, serine, and threonine were also detected in "hairy" root lines (Figure 1 and Figure 2). All spectral data obtained in this study correlate well with the data published earlier. In particular, the interpretation of ^{1}H and ^{13}C signals in the NMR spectra for fructose, glucose, and sucrose was published in (Skakovski *et al*., 2013). The proton and carbon spectra of galactose are available in (https://bmrb.io/metabolomics/mol_summary/show_data.php?molName=D_galactose&id=bmse000013). The assignment of inositol NMR signals as described in (https://www.chemicalbook.com/SpectrumEN_87-89-8_1HNMR.htm) for ^{1}H and in (https://www.chemicalbook.com/SpectrumEN_87-89-8_13CNMR.htm) for ^{13}C. Inulin ^{1}H and ^{13}C signals detection were described by Cérantola *et al.* (2004) and Caleffi *et al*. (2015),

respectively. Chemical shifts in the spectra of ^{1}H and ^{13}C γ-aminobutyric acid, alanine, asparagine, valine, leucine, lysine, proline, serine, and threonine were given in (Skakovskii *et al*., 2014); glutamine and isoleucine – in (Shysh *et al*., 2017). Spectra of arginine hydrochloride (^{1}H and ^{13}C) were described in (https://www.chemicalbook.com/SpectrumEN_32042-43-6_1HNMR.htm) and (https://www.chemicalbook.com/SpectrumEN_1119-34-2_13CNMR.htm).

Figure 1. ^{1}H NMR (A) and ^{13}C NMR (B) spectrum of D_2O extract of the roots of the control *A.annua* plants

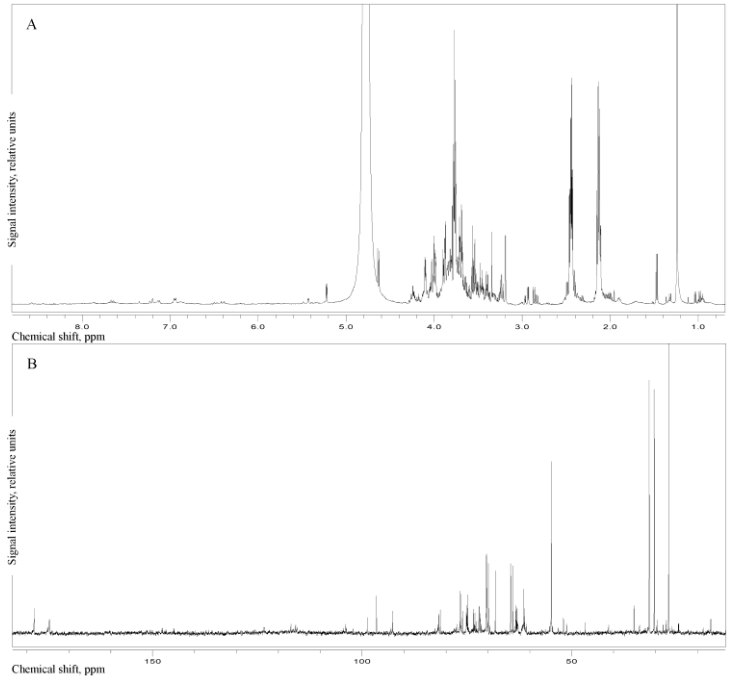
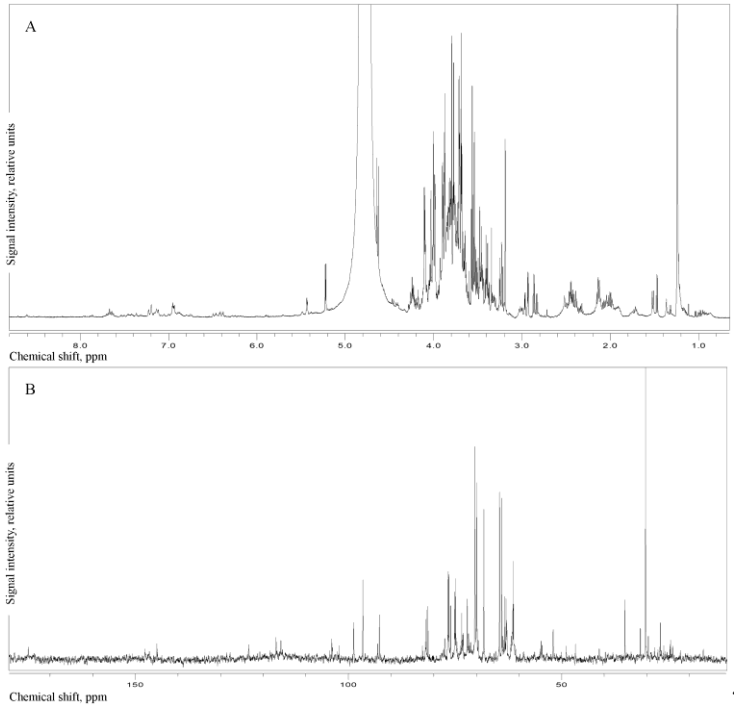


Figure 2. ^{1}H NMR (A) and ^{13}C NMR (B) spectrum of D_2O extract of *A. annua* "hairy" root line No3



Analysis of lyophilized samples of *A. annua* (Table 1) showed that spectra of the samples were generally qualitatively similar to each other and contained a set of signals of different carbohydrates and amino acids. Significant differences in the integrated intensities of the lines indicated a different quantitative composition of the studied extracts.

Analysis of the spectra of the studied extracts from the leaves and roots of control plants showed that they differ in the composition of carbohydrates and amino acids. Thus, the content of glucose and inositol in the leaves of control *A. annua* plants was 1.70 and 2.03 folds higher than in the roots. At the same time, the content of sucrose and fructose in the roots of control plants was 1.76 and 1.70 folds higher than in the leaves, respectively. Galactose was absent in the roots, but it was found in the leaves of the control plants.

Comparison of the carbohydrate content in the control and transgenic roots revealed a number of differences. In particular, the transgenic roots did not contain inositol (Table 1) but this compound was identified in the control roots (0.92%). Transgenic roots of *A. annua*, as well as roots of the control plants, did not contain galactose, although the leaves of control plants accumulated a small amount of galactose (0.84%). In all lines of "hairy" roots, the content of inulin increased in comparison to the control in 1.52 - 1.86 folds. Sucrose content in the “hairy” root lines differed from 0.42 to 1.17% and was lower compared to the control in the “hairy” root line No 1 and greater in line No 3. Similar data were obtained in the study of fructose content in “hairy” roots. In this study, root lines No1 and No2 accumulated fructose in an amount lower than in the control roots. At the same time, the content of fructose in root line No 3 was 1.24 folds greater than in the control roots. Thus, genetic transformation affected the level of sugar accumulation in the “hairy” roots of wormwood. This effect was expressed in the increased content of some compounds (for example, inulin) in all of the “hairy” root lines compared to the control roots. In our previous study, we evaluated sugar content in *A. vulgaris* and *A. dracunculus* “hairy” roots (Drobot *et al*., 2017). The results of this study demonstrated that sucrose content was 1.6 folds higher in *A. vulgaris* “hairy” root lines compared to the roots of the control plants. Fructose content was found to be 3.4 folds higher in *A. dracunculus* “hairy” roots than in the control roots. So, *A. rhizogenes*-mediated transformation really can affect sugars synthesis in *Artemisia* spp. “hairy” roots.

Analysis of the amino acid content in the roots and leaves of control plants revealed significant variability. In particular, alanine, asparagine, valine, isoleucine, lysine, threonine predominated in the leaves of plants. For example, the leaves contained 2.5 folds more arginine HCl than the roots. At the same time, roots accumulated more glutamine than leaves. Tryptophan and phenylalanine were not detected in *A. annua* control plants (both in the roots and leaves).

The total amount of amino acids (in %) in “hairy” roots was lower compared to the leaves of the control plants. Tryptophan and phenylalanine were found in one of the lines of “hairy” roots, while in control plants and other lines of “hairy” roots they were not detected. The content of asparagine in the "hairy" roots was 1.8 - 3.8 folds higher than the content of the compound in the roots of the control plants. It is of special interest that lysine was detected in all studied “hairy” root lines (0.18-0.32%) and it was absent in the control roots.

Table 1. The content of carbohydrates and amino acids in *A.annua* control plants (roots and leaves) and "hairy" root lines

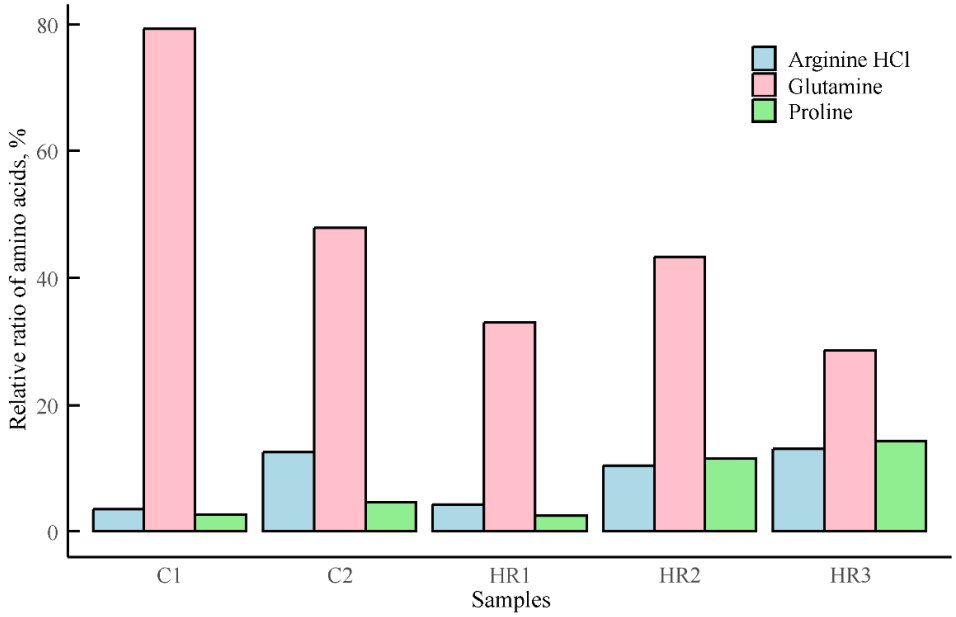
Compound	Carbohydrates and amino acids content, %				
	Control		Transgenic lines No		
	roots	leaves	1	2	3
Galactose	–	0.84	–	–	–
Glucose	2.33	3.98	2.61	2.33	2.95
Inositol	0.92	1.87	–	–	–
Inulin	1.01	1.15	1.54	1.63	1.88
Sucrose	0.72	0.41	0.42	0.77	1.17
Fructose	6.61	3.87	2.44	4.62	8.19
Σ (defined carbohydrates)	11.59	12.12	7.01	9.35	14.19
γ-aminobutyric acid	0.16	0.30	0.18	0.16	0.19
Alanine	0.29	0.41	0.06	0.16	0.19
Arginine HCl	0.40	1.00	0.24	0.64	0.69
Asparagine	0.68	0.96	2.61	1.23	1.30
Valine	0.11	0.18	0.02	0.03	0.03
Glutamine	8.95	3.83	1.82	2.65	1.51
Isoleucine	0.05	0.26	0.02	0.02	0.03
Leucine	0.06	0.08	0.01	0.04	0.04
Lysine	–	0.34	0.18	0.29	0.32
Proline	0.30	0.37	0.14	0.70	0.76
Serine	0.20	0.08	0.08	0.09	0.09
Threonine	0.10	0.19	0.08	0.09	0.14
Tryptophan	–	–	0.04	–	–
Phenylalanine	–	–	0.04	–	–
Σ (defined amino acids)	11.30	8.00	5.52	6.10	5.29

The increased content of proline in two of the three lines of "hairy" roots was studied, and it was more than 2 folds higher than in the control roots. Also, the lines of "hairy" roots were characterized by a high content of asparagine. It is known that the metabolism of arginine, proline, and glutamine in plants is closely related and increases in response to stress (Yang *et al*., 2017). In plants, the biosynthesis of proline occurs in two ways, through glutamine and ornithine, and the accumulation of proline is significantly increased under stressful conditions. The biosynthesis of arginine from ornithine derived from glutamine has also been studied (Anwar *et al*., 2018). Arginine is a nitrogen-rich amino acid with high nitrogen:carbon ratio (4:6), which makes it suitable in the plant for nitrogen storage during aging and seasonal changes. Arginine catabolism is associated with nitrogen remobilization and also plays an important role in developmental processes, especially in germination. Catabolism of arginine in mitochondria is the main source of endogenous urea in plants, as urea recirculation is very important for plant survival under stress (Goldrai and Polacco, 2000). Thus, arginine is involved in increasing the resistance of plants to abiotic and biotic stresses. If we analyze the distribution of these three amino acids in *A. annua* samples, taking the total content of identified amino acids as 100%, a noticeable decrease in the glutamine content will be found against the background of an increase in arginine hydrochloride and proline in the hairy roots of *A. annua*. (Figure 2). It can be assumed that the increase of the content of arginine hydrochloride and proline was initiated by the process of the transformation by *A. rhizogenes* known as phytopathogenic bacteria.

Thus, the genetic transformation has led to changes in the accumulation of amino acids (both quantitatively and qualitatively). We identified the "hairy" root lines, the content of individual amino acids in which significantly exceeded their content in the control.

The differences in amino acid content in "hairy" root lines compared to the control were studied by Gabr *et al.* (2019). Authors found elevation of the concentration of semi-essential amino acids such as lysine, isoleucine, valine, histidine, and phenylalanine in the "hairy" roots of common buckwheat. In this study, the content of proline increased 3–5 folds in "hairy" root lines compared to the control. In transgenic *Camellia sinensis* (L.) Kuntze roots content of L-theanine was 23% higher than in the roots of the control plants (Zhang *et al*., 2015). Authors associate this effect with *A. rhizogenes*-mediated transformation.

Figure 3. The relative ratio of arginine hydrochloride, glutamine and proline content in the control plants and lines of "hairy" roots of *Artemisia annua*: C1 – control roots; C2 – control leaves; HR1, HR2, HR3 – "hairy" root lines No 1, No 2, and No 3



4. CONCLUSION

The composition of carbohydrates and amino acids in three lines of "hairy" roots of wormwood in comparison to the control plants cultivated *in vitro* was studied. Inulin content in transgenic root lines was greater compared to the control plants, and this increase was more than 30%. The "hairy" roots lines, in contrast to control plants, did not contain inositol. The decreased glutamine and increased arginine hydrochloride and proline content in transformed root lines were detected. It was found that the largest amount of carbohydrates (14.19%,) was found in the "hairy" root line No 3, and the lowest (7.01%) - in line No 1. These parameters were higher and lower respectively compared to both controls. The highest total amino acids content (6.10%) was found in sample No 2. Seven of the total identified amino acids are indispensable amino acids: valine, isoleucine, leucine, lysine, threonine, tryptophan, and phenylalanine. The data obtained demonstrated that the genetic transformation of *A. annua* plants has led to the changes in the accumulation of carbohydrates and amino acids (both quantitatively and qualitatively). "Hairy" root lines with increased content of individual carbohydrates and amino acids were identified. The effect of stimulation of biosynthetic capacity of the cells of "hairy" roots after the transformation of *A. annua* plants by *A. rhizogenes* can be used for obtaining the root lines – producers of valuable bioactive compounds.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Hanna Shutava: Investigation, Writing-original draft. **Lyudmila Tychinskaya:** Formal analysis. **Eugeny Skakovskii:** Formal analysis. **Volodymyr Duplij:** Editing, Review, Visualization, Validation. **Yakiv Ratushniak:** Plants and "hairy" roots subcultivation, preparing plant material for the study. **Nadiia Matvieieva:** Investigation, Writing-original draft, Writing-review & editing**.**

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Assessment of the Impact of Climate Change on Forestry in Azerbaijan

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Abstract

While it is not possible for humans to influence climate change on Earth, the assessment of climate change in ecosystems is also one of the global challenges of our time. It should be noted that forest ecosystems cover the world's largest surface carbon pool. Climate change observed in the Republic of Azerbaijan plays an important role in the structure and function of forests in the Greater and Lesser Caucasus. The main task of the study was to assess forests using dendrochronological studies to study the exposure of forests to environmental changes in the Greater and Lesser Caucasus. The age of older specimens in the forests was determined, and the dendroclimatological effect of climatic factors on the forest cover over the years was analyzed. The article examines the impact of carbon dioxide on plant growth dynamics, phenology, growth, root system, productivity due to climate change in the Greater and Lesser Caucasus in Azerbaijan in recent decades on a scientific basis.

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1. INTRODUCTION

The Earth's climate is a complex, multi-component system that has undergone many changes throughout history. One of the main causes of global variability in the atmosphere in the 20th century is the influence of anthropogenic factors in the environment (Abrams and Orwig, 1995). Currently, climate change is one of the most discussed topics in our country. According to a recent assessment by the Intergovernmental Panel on Climate Change (IPCC, 2009), global average temperatures have risen by 0.8-1.2 °C over the past 100 years. The reason for the increase in temperature in our country is due to economic development, increase in transport pollutants and other anthropogenic factors. The basis of anthropogenic factors is gases that create a thermal effect: CO_2, CH_4, N_2O, SO_2, Cl_2 and Br_2 compounds (Adam *et al*., 2009). This study analyzes the impact of these or other factors on forest cover in the Greater and Lesser Caucasus. The study was conducted in accordance with the UN Framework Convention on Climate Change (UNFCCC). Studies have shown that climate change in the Greater and Lesser Caucasus and the impact of human activities on forest vegetation lead to drought, stress and partial climate change.

2. MATERIAL and METHODS

The historical development path of plant species *Pinus eldarica* Medw., *Carpinus betulus* L., *Quercus castaneifolia* C.A.Mey in the forest ecosystems of the Greater and Lesser Caucasus allows collecting information about exogenous events, factors influencing the potential growth of trees. For this purpose, samples were taken from plant specimens in the form of pens and discs. Dried samples with a height of 0.5-1.6 meters, a diameter of 4-5 mm and a length of 10-

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50 cm were transferred to laboratory conditions, and chronological data were collected using SUUNTO and MAKITA devices. Through TSAPWin and ARISTAN programs, the width of the annual rings was measured with a binocular microscope to the nearest 0.01 mm and coding was recorded on the samples. Data on changes in the rings of plants every 10 years were collected. Crossdating was used in the TSAPwin program to identify climate change in the species (Angert *et al*., 2005). Temperature, amount of precipitation, number of sunny days in the country were analyzed on the basis of statistical data provided by the Statistics Committee of the Republic of Azerbaijan, the Ministry of Ecology and Natural Resources (Badeau *et al*., 1996). Based on the annual rings of the samples, data on climate change in the past were calculated graphically using Lintab 6 and Resistograph dendrochronological equipment. Data quality results and annual ring series were evaluated by the Crossdating method according to COFECHA program (Baker, 2004). The similarity between the positive (upper) and negative (lower) inclinations of the annual rings was determined by the Gleichläufigkeit (GLK) method (Barber *et al*., 2000) to determine the parallel correspondence. Based on the COFECHA program (Barnett *et al*., 2005), the correlation coefficient of the series was determined with 99% accuracy. If the correlation coefficient falls below 0.3281, this threshold is considered a critical level.

3. RESULTS and DISCUSSION

The recent rise in temperature in the world is due to anthropogenic factors. These anthropogenic factors are formed on the basis of the thermal effect and consist of carbon, methane, nitric oxide, nitrogen monoxide and chlorine-fluorine gas compounds. At the same time, the intensity and frequency of hot winds, hurricanes, rains and floods in nature have increased significantly in the last 10 years. Earlier, the sea and ocean waters were heated to a depth of 1.000 meters, but recently the temperature has risen to a depth of 2.000 meters. These warm currents have a negative impact on biodiversity in the oceans and seas, which is a key indicator of natural disasters. The increase in temperature caused by global climate change has led to the melting of snow and ice in the regions, and the rise of the global Mediterranean level (IPCC, 2009).

In recent years, the Laboratory of Dendrochronology of the Institute of Dendrology of ANAS conducts extensive research to address these or other problems caused by climate change. Dendroclimatological research conducted by the staff of the Institute analyzed the impact of climate change on tree growth in forests. This allows for the collection of long-term data on forest development and annual tree growth (Abrams and Orwig, 1995). It should be noted that the Republic of Azerbaijan has not been left out of the impact of global climate change. Over the past 100 years, the average annual temperature in Azerbaijan has increased to 0.4-1.3 °C. The temperature in the Greater and Lesser Caucasus rose by 0.2-0.9 °C. The highest increase was observed in the lowlands, the lowest in the mountains. This temperature increase is unevenly distributed depending on the region. During the last 15 years, the average monthly temperature in these areas was higher than the climatic norm for 11 years (2004, 2005, 2007, 2010, 2011, 2013, 2014, 2015, 2016, 2017, 2018). The average monthly temperature was 7 years (2005, 2006, 2008, 2011, 2012, 2014, 2017) below the climatic norm in February. The average monthly temperature was higher than the climatic norm for 8 years (2004, 2009, 2010, 2013, 2016, 2018, 2019) in July-August (stat.gov.az). During the research, the number of cloudy days and windy days in the country has recently decreased by 15-20 days a year.

Table 1. The following table shows the different number of sunny days in the country.

	number of sunny days per year (days)
Greater Caucasus	12-14
Lesser Caucasus	5-6

As can be seen from Table 1, the number of sunny days per year in the Lesser Caucasus was higher than in other areas. In the Greater Caucasus: Sheki-Zagatala, Guba-Khachmaz zones, on the contrary, the number of sunny days was less than the norm (Table 1). The number of cloudy days has increased in those areas. The study found that the amount of precipitation in the Lesser Caucasus decreased by 15-80 mm (Table 2).

Table 2. The amount of precipitation in the Greater Caucasus (GC) and Lesser Caucasus (LC).

By zones in the republic	The amount of precipitation
In the Upper Karabakh zone (LC)	30-38
In Aghdam (LC)	85
Gadabay (LC)	88-181
In Neftchala (LC)	221
In Guba, Gusar, Gyriz, Khachmaz zone (GC)	74-90
Quba 150, (GC)	
Balakan, Zagatala, Sheki, Oguz, Gabala, Ismayilli, Shamakhi, Altiagaj (GC)	25-89
In the Central Aran regions	32-89
In Beylagan	113

As can be seen from Table 2, the amount of precipitation was higher in the Lesser Caucasus. In the lowlands of Azerbaijan, the reduction in precipitation would not have had much of an impact on its geographical location. However, since Azerbaijan is located in both plains and mountains, these changes create many complications. Thus, the area of moisture-loving plants in the country is decreasing, the boundaries of the landscape in mountainous areas are changing, the boundaries of humid zones are falling, the boundaries of thermal zones, as well as the lower limit of our forests are rising (eco.gov.az).

During the year, the amount of precipitation in the mountainous zone of the Lesser Caucasus varies depending on the altitude. Although the amount of precipitation decreased throughout the country, it is registered the increasement of the amount of precipitation in the Sheki-Zagatala region of the Greater Caucasus by about 10 mm on average. It should be noted that the impact of this climate change leads to many problems in the process of adaptation of plants in forests. A certain drop in temperature increases the role of precipitation in humidity. Decreased rainfall does not meet the plant's need for water during growth and transpiration. Changes in the amount of sunny days and precipitation in the last 100 years have a negative impact on biodiversity in different regions of Azerbaijan, increasing the number of floods in mountain rivers, public health, food security, tourism and resort recreation. It should be noted that Azerbaijan is a mountainous country, the spatial and temporal distribution of natural factors, agro-climatic indicators also depend on altitude and indented terrain. At the same time, the country is located in the southern hemisphere, the areas receive a lot of sunlight and heat. Thus, in the Lesser Caucasus, the duration of sunlight is 2200-2400 hours per year, and the annual amount of FFR (photosynthetic active radiation) is more than 64 kcal/ cm^2. The maximum effect of these indicators is observed more in the Arzaboyu plain (more than 2800 hours and more than 76 kcal / cm^2, respectively). The effect of climatic factors on plant

development in forest species using international distances in tree species was analyzed (Fritts, 1976).

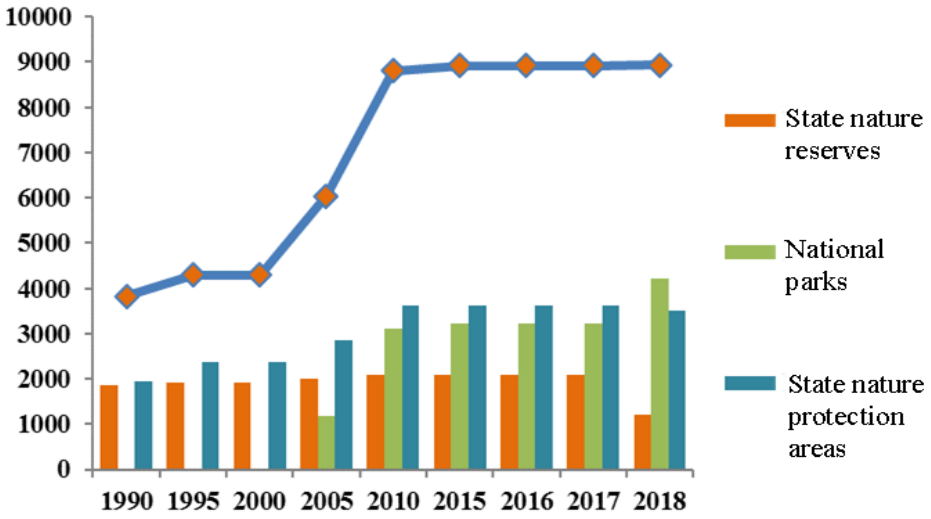
Studies have shown that rising temperatures and variable rainfall in forests have affected forest growth and productivity. Thus, the impact of climate change in the northern regions of the Greater Caucasus (Guba, Khachmaz, Gabala, etc.) has led to a rise in temperature of 1.2 °C, which led to large-scale landscaping (Brown *et al*., 2004) led to the adaptation of Mediterranean ornamental plants to local conditions. Climate change in the late twentieth century has led to increased crop yields in some parts of the Greater Caucasus. In some areas, on the other hand, the sensitivity of the temperature reaction has led to their weakening, reduced growth and degradation of areas. In order to increase biodiversity, new national parks, reserves and sanctuaries have been established since 2003 in accordance with the President's decree, which is important for the restoration of natural flora in the country (Table 3 and Figure 1).

Table 3. The area of specially protected lands. Specially protected areas (km^2) (eco.gov.az).

	2005	2010	2015	2016	2017	2018
Total area of specially protected lands	6038.2	8807.7	8925.5	8925.5	8925.5	8928.6
including:						
State nature reserves		2090.8	2090.8	2090.8	2090.8	1207.2
National parks	1177.5	3105.3	3223.1	3223.1	3223.1	4213.7
State natue protection areas	2851.8	3611.6	3611.6	3611.6	3611.6	3507.7
Percent ratio (%) to the total area of the country	7.0	10.2	10.3	10.3	10.3	10.3

Date of last update of indicators: April 29, 2019

Figure 1. The changes in the area of specially protected land.



As can be seen from the Table 3, the area of specially protected lands in 2005 was 6038.2 ha, while in 2018 it reached 8928.6 ha. The role of specially protected areas in the conservation of biological diversity is irreplaceable. By the end of 2018, 10 state nature reserves with a total area of 120.7 thousand hectares, 10 national parks with an area of 421.4 thousand hectares and 24 state nature reserves with an area of 350.8 thousand hectares for the preservation and reproduction of flora and fauna in the country were established. Currently, specially protected natural areas make up 10.3% of the country's territory.

According to statistics, the main indicators of the country's forest fund were analyzed, and it was found that the growth of artificial forests on unusable agricultural lands in the forest fund increased from 65.000 hectares in 2005 to 113.000 hectares in 2018 (eco.gov.az). At the same time, the gases emitted from stationary sources into the country's atmosphere contribute to the increase in CO_2 and nitrogen sediments in forests, the physiology of plants, had a positive effect on growth (eco.gov.az) (Table 4).

Table 4. Gases from stationary sources that emitted into the country's atmosphere and created a thermal effect. (thousand of tons)

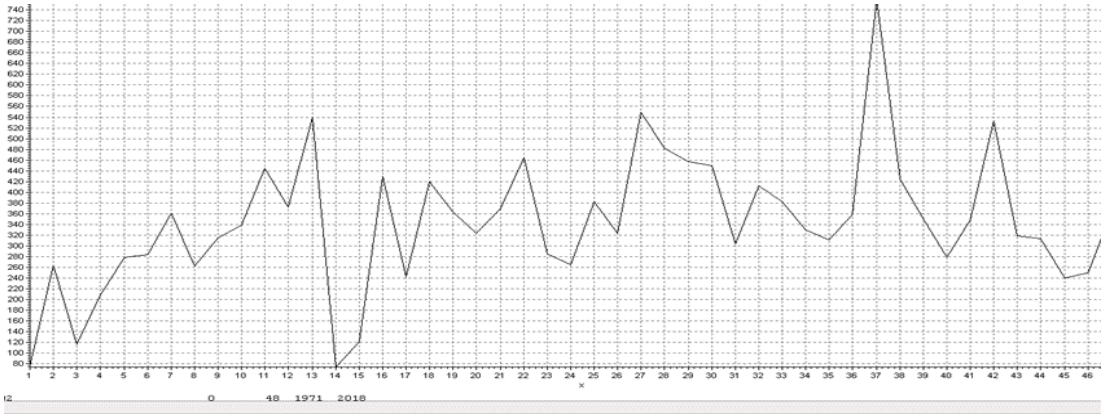
	2007	2009	2010	2012	2014	2016	2018
Carbon dioxide (CO_2)	14828.8	15293.1	14399,6	12471,4	16091,9	14911,2	18494,1
Nitrogen monoxide (NO)	1.7	10.4	11.8	15.8	4.7	3.3	8.7
Methane (CH_4)	24.3	24.2	18.3	385.2	95.7	41.0	18.2
Hydrofluorocarbons	0.5	7	6.8	6.4	1.8	0.8	0.6
Sulfur hexafluoride (SF_6)	0.1	0.6	0.3	0.6	0.05	0.03	0.004
Perfluorocarbons	0.6	6.4	5.6	5.6	0.02	0.01	0.01

The increase in rainfall led to an increase in growth and forest areas (Makinen *et al*., 2003). In some areas, excessive CO_2 levels have led to the onset of droughts in forests. At the same time, although climate change affects the physiology and growth of trees, an increase in ozone in the area counteracts and balances the positive effects of CO_2. In addition, rising temperatures in arid areas do not directly affect soil water, but they do cause stress. As a result of the recent impact of stress, there is a shortage of water in many regions of the country, including many wells in Absheron (Borgaonkar *et al*., 2009).

Although a small increase in temperature leads to photosynthesis and tree growth, excessive temperatures prevent this (Way and Oren, 2010). In the Lesser Caucasus, since the middle of the 20th century, the excessive temperature in the forests has led to an increase in density, while temperature sensitivity has led to an anomalous decrease (Brown *et al*., 2004). As a result of the research, the climate change observed in recent years, in addition to causing warming, has had a positive effect on reducing the amount of solar radiation, the process of photosynthesis and the growth of plants. Increased CO_2 levels and nitrogen (N) precipitation in forests is the result of anthropogenic factors.

Dendrochronological analysis of older specimens in the Greater and Lesser Caucasus compared the favorable years with the unfavorable years in the trees in the area and collected information about the historical period of its passage on the basis of wide and narrow ring patterns. The relatively fast and slow growth of trees is called cross-adaptation to the rings. By linking this to climate data, the role of climate change in tree growth was analyzed. Exact calendar years have been determined by the method of crossing tree rings (Stokes and Smiley, 1968). Using climate data by year, dendroclimatological studies considered their resistance to annual influences and their geographical scale in synchronous interval variability. An extensive chronology of tree specimens (Hughes, 2002) was conducted, with models of growing and easily verifiable climate-growth relationships being established. In recent decades, climate change in the Republic has led to a significant increase in the width of the Eldar pine (*Pinus eldarica*) ring. An increase in CO_2 in mountainous areas is more common in the country. The mechanism of the impact of climate change on the dynamic development of the species *P. eldarica* as a result of the increase in CO_2 from 1971 to 2018 is shown in the diagram below (Figure 2).

Figure 2. Mechanism of influence of climate change on the dynamic development of *P. eldarica*.



It has been determined that the width of the annual rings of 80-100-year-old *Carpinus* species in Shahdag National Park has been larger in recent years than in 1850. This is explained by the fact that the increase in CO_2 observed in tree ring widths is due to an increase in biomass. This is due to the fact that plants grow faster due to increased CO_2 concentrations in the atmosphere, and accelerated growth leads to an increase in underground root mass and cambium activity, which leads to changes in tree-ring width (La Marche *et al*., 1984). Radial growth in plants in the southwest of the Greater Caucasus is influenced by atmospheric CO_2. Approximately 61% of the total change in the radial growth index is due to the climate of both young and old trees. In 2010-2019, the annual ring remnants of young trees developed more strongly than in 1900-1996. As a result, some tree species grow well in the early stages of development, and at the same stage of development, in areas where the concentration of CO_2 in the atmosphere increases, they have a greater growth response.

In the analysis of tree samples taken from trees 25-30 m high in the Lesser Caucasus, it was found that plant growth has been more intensive in the last decade. Higher growth has been observed in tree samples taken from the Gadabay zone over the past 50 years. This rapid growth in the forests of Azerbaijan indicates an unprecedented ecological change in recent centuries. The increase in temperature observed in the forests was reflected both in the growth interval and in the width of the ring. In the course of the research, it was found out that the increase in temperature in some areas of the Republic has played an important role in the level of species growth. This shows that the increase in temperature in our country was in the direction of a significantly increasing trend compared to the last century. *Carpinus betulus, taken* in the Shahdag National Park of the Greater Caucasus, has seen an abnormally high increase in ring width chronologies over the past few decades. These chronologies show that the same year is associated with average annual and winter temperatures (Borgaonkar *et al*., 2009). *C. betulus* species high in 1972, 1974, 1977, 1982, 1983, 1984, 1994, 1997, 2001, 2007, 2012, 2019. Low rates were observed in 2010, 2015, and 2018. The increase in CO_2 in the Great Paper is more pronounced in the species *Q. castaneifolia*. In forests, trees grow up to 12 % faster than surrounding plants. Warming in forests in the northern regions of the Greater Caucasus in the 20^{th} century led to faster plant growth than in 1850, especially as rising CO_2 levels had a greater impact on the growth and development of young plants. *Q. castaneifolia* species under the influence of climatic factors in 1960, 1967, 1969, 1994, 2006, 2016 showed low species performance, high growth dynamics compared to 1964, 1970, 1979, 1985, 1987, 2000, 2002, 2012 was observed (Figure 3). It has been found that factors of anthropogenic origin play an important role in determining the history and development of plants. At the same time, harmful gases emitted into the atmosphere from stationary sources are a major factor in rising temperatures and climate change. The mechanism of influence on the dynamic development of

Q. castaneifolia as a result of climate change and increase in CO_2 from 1971 to 2015 is shown in the diagram below (Figure 4).

Figure 3. Dendroclimatological study of *Q. castaneifolia*.

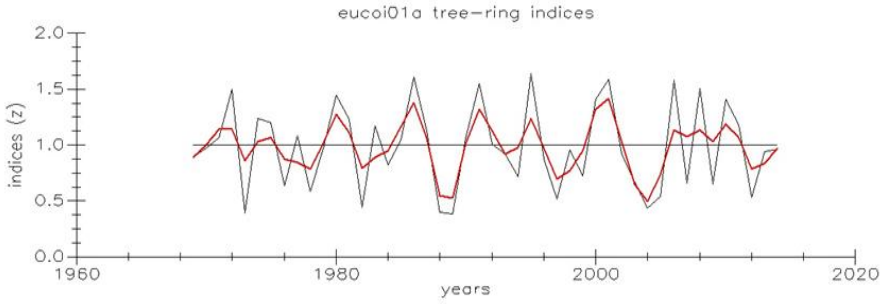
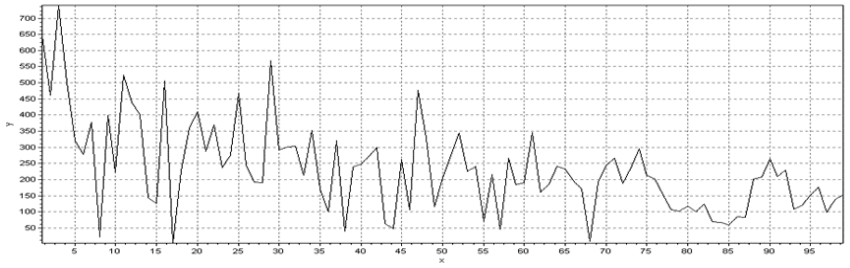


Figure 4. The mechanism of influence of climate change on the dynamic development of the species *Q. castaneifolia* over the years.



Thus, fourteen chronological ring width data have been used to model low-frequency temperature variability in the study of the effects of anthropogenic factors on plants over the past centuries. Data from these fourteen locations were taken from the northern (eight-barrel area, 55°-70° N) and southern (six temperate areas, 30°-55° N) areas of the Greater Caucasus. It was found that in the northern zone there has been a recent decline, while in the southern zone the warming tendencies are higher. In the southern regions, a decrease of more than 30 % compared to the northern regions, in the range of 5-12 % was observed. It can be concluded that the gases emitted into the atmosphere of the country from stationary sources in the Republic are expected to affect the environment in different directions, creating global climate change (IPCC, 2009). Thus, in the northern regions, the projected warming will lead to drought in forests (Hogg and Hurdle, 1995), which could result in fires (Flannigan *et al*., 1998). Recent climate change and increasing drought stress in the Lesser Caucasus is expected to lead to a decrease in tree growth in some highlands (Barber *et al*., 2000). This climate change may have a negative impact on forest cover in arid areas. In addition, climate change will lead to the emergence of new species of pests, including both native and exotic species (Krcmar-Nozic *et al*., 2000). The combined effects of drought and fire can result in an increase in forest fires. Early snowmelt in winter and spring in these areas allows us to predict new disasters. As a result of warming in our country, the melting of snow in the mountain forests of the northern regions can lead to drought at the end of the season, causing strong evaporation. Water supply will depend on snowmelt during the winter (Barnett *et al*., 2005; Adam *et al*., 2009). Water in ponds will increase in the spring (Barnett *et al*., 2005; Xu *et al*., 2008) and will reduce the available reservoir for active tree growth. Increased temperature sensitivity in trees that grow faster in areas can lead to drought stress. In recent years, there has been a decline in tree growth

in the Lesser Caucasus. Decreased growth is more common in hot and dry areas, and increasing warming in recent decades may lead to increased drought stress in areas. According to statistics, warming is more affected by drought stress in the same areas.

The reason for the decline in growth in the Greater Caucasus is due to drought and pests. In addition, in late winter or spring, drought or pathogenic insects cause great damage to tree roots, which can result in the mass destruction of many plants. Drought stress caused by warming has led to an increase in regional forest area (BAI) in recent years, especially in young trees (Wu *et al*., 2013). The increase in temperature has a negative impact on the growth of plants in the temperate semi-arid forests of the Lesser Caucasus. As a result of the hot spring, there are restrictions on tree growth. A dendroclimatological study of oak species in the Lesser Caucasus found that radial growth was limited by humidity in the summer. Growth is observed in weak dry soils in spring and in September-October. However, research has shown that increasing CO_2 concentrations and temperature rises in forests have a significant effect on tree growth. It has caused changes in the population, survival, productivity and phenological development phases of species in forests. Tree ring analysis conducted in different regions with reduced crop yields showed that the effect of climate change on the growth of plant areas. In different areas, the change in ring width was similar for different trees for some time, but then their ring width had completely different characteristics. These climate changes also affect the soil characteristics of the area.

4. CONCLUSION

In this study, the effects of carbon dioxide concentration and temperature changes caused by climate change in the Greater and Lesser Caucasus on the growth and productivity of plants in that region were investigated. As a result of the study, it was revealed that CO_2 concentration and increases in temperature have a significant effect on tree growth. It has been determined that as a result of climate change, trees affect productivity and soil structure.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

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The Impact of Cetuximab on Apoptotic and Autophagic Gene Expression in Metastatic Colorectal Cancer Cells

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Abstract

Colorectal cancer (CRC) is one of the most frequent cancers, and chemotherapy plays an important role in its treatment. The primary goal of this research was to investigate the apoptotic, autophagic, and cytotoxic effects of cetuximab (CTX), a monoclonal antibody used to treat colorectal cancer. Colorectal cancer cell lines were treated with different doses of cetuximab to create ten test groups, which were then incubated for 24 and 48 hours. The cytotoxicity was determined using the MTT test. Histochemically, the TUNEL assay was employed to identify apoptosis. Real-time PCR was used to determine the expression levels of *p21, p27, p57, KRAS, LC3A, BECN1, EGF*, and *ATG4A* genes. The effective dose of cetuximab was determined by the MTT cytotoxicity study to be 10 g/mL. *KRAS and EGF* gene expression decreased at the same time, but *p21, p27, p57, LC3A, BECN1*, and *ATG4A* gene expression increased. With increasing doses of cetuximab, an increase in apoptosis was identified using TUNEL labeling. We may conclude that CTX induces apoptosis and autophagy in HT-29 KRK cells through raising the expression of *p21, p27*, and *p57* genes, as well as *ATG4A, LC3A*, and *BECN1* genes. Furthermore, it is possible to say that reducing *EGF* and *KRAS* gene expression inhibits cancer cell growth.

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1. INTRODUCTION

Colorectal cancer (CRC) is the third most common type of cancer among men and women worldwide (Torre *et al*., 2016). Cetuximab (CTX), an epidermal growth factor (EGF) antagonist monoclonal antibody, is widely used in the treatment of CRC. CTX can reduce the activation of the receptor and activate apoptosis by inhibiting the binding of the ligand to the relevant receptor in certain cancer cells. It has been shown that the combination of anti-epidermal growth factor receptor (a-EGFR) treatment and cytostatics that cause DNA damage causes an antitumoral effect by inhibiting cell cycle progression and activating apoptosis (Shao *et al*., 1998). EGFR overexpression is variable in the pathology samples of colorectal cancer cases and is detected in approximately 25–82% of the cases (McKay *et al*., 2002). Cetuximab increases apoptosis while suppressing cell proliferation, angiogenesis, and metastasis by blocking downstream signaling. Due to its immunoglobulin G1 (IgG1) structure, it also activates immune effector cells that provide antibody-dependent cellular cytotoxicity (Mendelsohn & Baselga, 2003).

Apoptosis is a genetically controlled cell death that ensures the safe destruction of cells that have lost their function, overproduced, aged, irregularly developed, and damaged DNA

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(Luporsi-Gely *et al*., 2001). Cancer occurs when the homeostatic balance between cell division and apoptosis is disturbed. Cyclin-Dependent Kinase Inhibitors (CDKI) are responsible for a negative control of the cell cycle (Tsihlias *et al*., 1999). Cell cycle progression is controlled by cyclin-dependent kinases (Cdk), which are the catalytic partners of cyclins that maintain the cycle. Cdk activity that is not regularly controlled causes increased cell proliferation and genomic instability. This situation results in cell immortality or cancer. Three different Cip/Kip (Cdk Inhibitory Protein/Kinase Inhibitory Protein) family CDKIs have been identified that regulate Cdk activity and stimulate cell cycle suppression. These are p21Cip1 (CDKN1A), p27Kip1 (CDKN1B), and p57Kip2 (CDKN1C) (Zieske, 2000). According to the results of research conducted in recent years, it is known that p21 induces growth arrest, p57 maintains growth arrest and p27 stimulates differentiation genes (Deschenes *et al*., 2001).

Autophagy means self-eating and it is used to mean that cells break down structures within the cell to obtain nutrients in the physiological state of starvation. In some studies, it has been seen that autophagy plays a role in metabolism, morphogenesis, differentiation, aging, cell death, and the immune system (Shintani & Klionsky, 2004; Mizushima *et al*., 2008). The relationship between cancer and autophagy is still controversial, and while autophagy acts as a tumor suppressor during cancer formation, it contributes to the survival of cancer cells during cancer progression (Levine & Kroemer, 2008; Yang & Klionsky, 2010). It is understood that autophagy plays an important role in both carcinogenesis and cancer cell survival. Cytotoxic and targeted drugs used in cancer treatment are known to induce autophagy. As a result, inhibiting or activating autophagy may have considerable therapeutic benefits in cancer treatment (Rouschop & Wouters, 2009). Kirsten Rat Sarcoma (Ki-or K-Ras), a viral oncogene homolog of the Kras gene, is a member of the Ras gene family. The Kras protein, a small GTPase, consists of 189 amino acids and plays a key role in Ras/Raf/MAPK signal transduction, which is involved in many pathways such as proliferation, differentiation, and apoptosis (Adjei, 2001).

The goal of this work was to look at the effects of CTX, a commonly used chemotherapeutic agent, on *p21, p27, p57, KRAS, LC3A, BECN1, EGF*, and *ATG4A* in HT-29 CRC cancer cells, as well as their interactions.

2. MATERIAL and METHODS

2.1. Cell Culture, Passaging of Cells, and Cell Count

The HT-29 cell lines were cultured in a DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) solution. Cultures were incubated at 37 °C with 5% carbon dioxide (CO_2) and 95% humidity. Cell culture studies were performed in the Bio Safety Cabinet (Class II). The medium of the cells that reached certain confluency (80%) was removed and washed with 4-5 mL of phosphate-buffered saline (PBS). Subsequently, 3 mL of trypsin/EDTA (Ethylene Diamine Tetraacetic Acid) was added. They were incubated in a CO_2 incubator during the period. 8 mL of fresh medium was then added and centrifuged for 5 min at 800 x g. The supernatant part was removed from the centrifuged cell suspension and 10 mL of fresh medium was added to the pellet. These passaged cell cultures were left to incubation at 37 °C in a 5% CO_2 incubator containing 95% humidity. 1µL of the cell suspension to be counted was transferred to a 0.5 mL eppendorf tube and 90 µL trypan blue dye was added to it and 10 µL was transferred to both chambers of the hemocytometer and the cell count was performed under an inverted microscope. Cell concentration was determined using the formula (1) below.

Number of cells / mL = Average number of cells in chambers × DF × 10^4 (1)

DF: Dilution factor.

10^4: Factor arising from chamber sizes on the slide (1 mm^3).

Thus, 5x10^6 cells were transferred to each 100 mm^2 culture petri dish and 5x10^3 cells to each well of the 96 well culture plate.

2.2. Drug Treatment

CTX (Cetuximab, Rhone-Poulenc Rorer Pharmaceutical) was used to duplicate each sample in wells. CTX concentrations of 0.1 g/mL, 1 g/mL, 10 g/mL, and 25 g/mL were prepared and treated for each well with an equal number of cells, and they were incubated for 24 and 48 hours, respectively.

2.3. Cell Viability Measurement

The cytotoxic activities of the samples on existing cell lines were measured using the 3-(4,5-dimethyl thiazol-2-il)-2,5-diphenyl tetrazolium bromide (MTT) kit (Cell Proliferation MTT Kit, Roche, Cat No: 11465007001) according to the manufacturer's instructions. The water-insoluble MTT tetrazolium salt is reduced to the water-soluble orange-colored formazan compound by the mitochondrial enzyme activity in metabolically active cells, and the product was measured in a microplate reader at 450 nm. The percent cell cytotoxicity/viability curve was drawn against the applied dose, and the 50% suppressive concentration (IC_{50}) value was calculated with the logarithmic slope graph in the Microsoft Excel Program.

2.4. Histochemical Staining and TUNEL Assay

Inoculations were carried out in equal amounts (approximately 5x10^3 cells suspended in 100 µL medium) on the upper surfaces of the lam in each petri dish. CTX treatments were applied after the cells adhered to the lam. Following the end of the 24 and 48-hour incubation period, PBS was added and cell washing was performed after removing the medium from the petri dish. The cells adhered to the lam were fixed with methanol at -20 °C for 10 min. The lams were washed with PBS and dried.

Terminal Deoxytransferase Mediated Bio-dUTP Nick End Labeling (TUNEL) was used for histochemical detection of apoptosis. The TUNEL kit (Roche, In Situ Cell Death Detection Kit, TMR red, Cat No: 12156792910) was used for the staining process. Adherent cell staining was performed according to the experimental protocol included in the kit. The "Stereological Optic Fractionator Frame" method was used to compare the results of TUNEL staining between the groups. This assay indicates late-stage apoptosis. The assay was performed under the stereology workstation system (BioPrecision MAC 5000 controller system, Ludl Electronic Products, Hawthorne, NY) and stereology software (Stereo Investigator version 9.0, Microbrightfield, Rochester, VT) under a light microscope with attachment (Leica, Cambridge, UK). For the determination of apoptotic cells on HT-29 cell preparations, the method of "Unident Counting Frame and Fractionator" was used and the TUNEL positive cell density in each preparation belonging to all groups was calculated according to the following formula:

PCD = PCN / (FA x FN)

PCD; TUNEL positive cell density per µm² area.

PCN; TUNEL positive cell count.

FA; frame area (µm²)

FN; the number of frames

The data obtained is based on duplicate measurements for each group. Three preparations for each group were stained.

2.5. PCR Reaction Analyzes

The Roche MagNA Pure Compact RNA isolation kit was used for RNA isolation from cell culture samples. The experimental protocol in the kit was used. The prepared sample tube was loaded into the ROCHE MagNA-Pure-Compact DNA-RNA isolation device. Then, 50 µL of the sample was obtained by RNA isolation with the Roche MagNA RNA isolation kit. The amount and purity of isolated RNA were measured spectrophotometrically in the NanoDrop device. The concentration of mRNAs obtained was measured and diluted to 15 ng of all mRNAs during cDNA construction according to the mRNA concentration. 10 µL of the samples whose concentration was adjusted, 2 µL of Random Primer from the Transcriptor First Strand cDNA Synthesis kit and 1 µL of distilled water (for each sample) were placed in 0.2 mL PCR tubes. Then, denaturation was performed at 65 °C for 10 minutes in the Reverse Transcriptor PCR device. While this process was going on in PCR, the mixture was prepared to form cDNA from denatured RNAs.

2.6. Measuring *p21, p27, p57, KRAS, LC3A, BECN1, EGF,* and *ATG4A* Gene Expressions by PCR

Using the cDNAs of each sample, gene expressions (*p21, p27, p57, KRAS, LC3A, BECN1, EGF, ATG4A*) were detected by the Roche LightCycler 480 Real-Time PCR device. Based on the standards G6PD, the results of the device for *p21, p27, p57, KRAS, LC3A, BECN1, EGF*, and *ATG4A* were recorded.

2.7. Statistical Analysis

Statistical analysis was performed by One-Way ANOVA with Duncan's post hoc test after determining a homogeneous distribution using SPSS software version 20.0 (SPSS Inc. Chicago, IL, USA), and a *p*-value less than 0.05 was considered as a significant difference between the groups. Linear regression analysis was used to determine the correlation between *p21, p27, p57, KRAS, LC3A, BECN1, EGF*, and *ATG4A* variables. The significance between the correlations of dependent and independent variables was determined by means of Fisher's exact test.

3. RESULTS and DISCUSSION

3.1. MTT Analysis Results

In our study, the dose and time-dependent cytotoxic effect of CTX was investigated. In the MTT analysis of the HT-29 cell line in CTX application, a dose-dependent cytotoxic effect was observed after 24 and 48 hours of incubation. In the statistical analysis, it was determined that in the groups administered CTX at 10 M and 25 M concentrations, there was a significant decrease in comparison to the control group during the 24-hour incubation period. The least cell viability was observed in the group treated with 25µM CTX in both 24-hour and 48-hour incubations. It was also determined that the same dose was toxic for the HT-29 CRK cell line after 24 and 48 hours of incubation (Table 1).

According to a literature review, 100 g/mL CTX administered to various CRC cell lines such as LIM1215, GEO, and SW403 resulted in a 70% reduction in proliferation (Jhawer *et al*., 2008). In addition, it was reported that there was a 50% decrease in proliferation in Caco-2 and SW948 CRC cell lines at the same dose (Jhawer *et al*., 2008). Similar to our results, Farias and coworkers (2012) applied CTX to HT-29 cells in their study and reported that the most appropriate dose was in the 10 nM CTX group as a result of MTT evaluation.

Table 1. MTT results after CTX application in 24 and 48 hours incubation for all groups.

	24h	48h
Control	0.865±0.21[a]	0.601±0,28[a]
0.1 µg/ml CTX	0.809±0.25[a]	0.475±0.12[a]
1 µg/ml CTX	0.734±0.21[a]	0.333±0.08[b]
10 µg/ml CTX	0.580±0.19[b]	0.380±0.17[b]
25 µg/ml CTX	0.477±0.16[b]	0.260±0.08[b]

a and b indicates statistical difference between groups in the same column (p<0,05). All data are expressed as mean ± standard deviation (SD). Statistical analysis One-way analysis of variance was performed with Duncan's Test. Statistical significance was accepted as p<0.05.

3.2. TUNEL Painting Analysis Results

In our study, the apoptotic effect of CTX on HT-29 CRK cells was determined by the TUNEL method. As a result, when TUNEL positive cell densities were calculated, it was discovered that the control group had the lowest TUNEL positive cell density of the groups incubated for 24 hours, but apoptosis increased significantly in the groups given increasing quantities of CTX (Table 2). It was determined that TUNEL positive cell density increased significantly, especially in the groups administered 10 and 25 µg/mL CTX. In the groups where CTX was applied for 48 hours, the lowest value was found in the control group, and this value grew significantly in the groups where CTX was applied gradually. Accordingly, it was determined that the highest TUNEL positive cell density was again in the groups treated with 10 and 25 µg/mL CTX. According to these results, it can be said that CTX increases apoptosis in HT-29 CRK cells at these concentrations (Table 3).

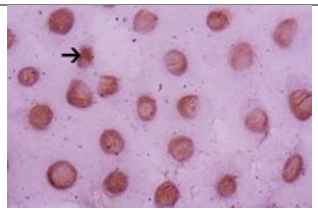
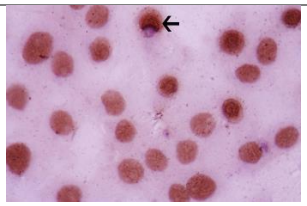
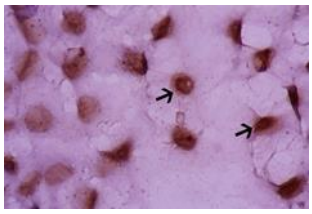
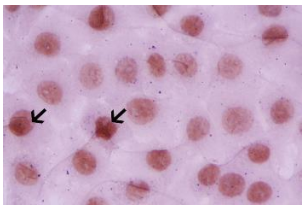
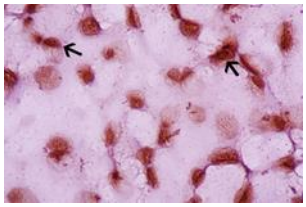
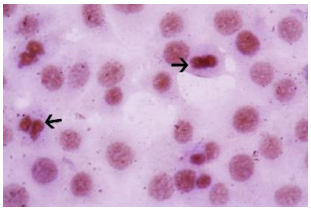
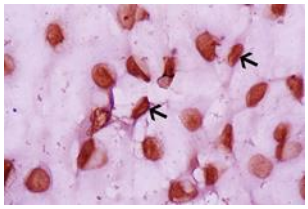
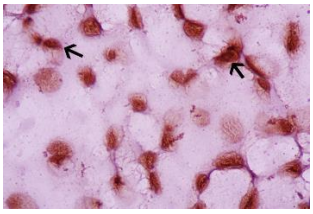
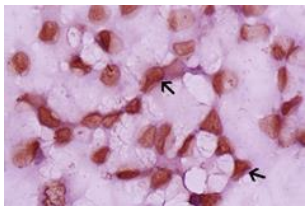
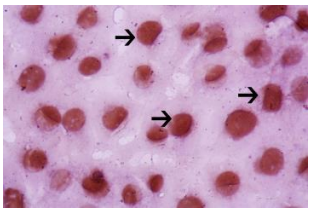
Table 2. TUNEL analysis results of the control and 0.1- 25 µg/ml CTX applied groups after 24 and 48 hours of incubation

	24h	48h
Control	1.14±0.32[a]	1.15±0.42[a]
0,1 µg/ml CTX	2.32±0.34[b]	2.91±0.32[b]
1 µg/ml CTX	2.52±0.45[b]	2.99±0.25[b]
10 µg/ml CTX	3.16±0.24[c]	3.44 ±0.29[c]
25 µg/ml CTX	3.46±0.27[c]	3.61±0.34[c]

a, b, and c indicates statistical difference between groups in the same column (p<0,05).

It has been reported that programmed cell death increased with CTX treatment in DiFi human CRC cells with high EGFR expression (Wu *et al*., 1995). In another study on CRC cell lines, it was reported that anti-EGFR antibodies caused an increase in Bax expression (Mandal *et al*., 1998), and another study reported that multiple apoptotic caspases were activated (Liu *et al*., 2000). Similarly, it was reported by Huang and coworkers (1999) that treatment of SSC cell lines with CTX caused an increase in Bax expression and a decrease in Bcl-2 expression, and also an increase in apoptotic cell frequency was observed. However, a report suggested that the use of CTX alone in most tumor cell lines is not sufficient to induce apoptosis and generally has a cytostatic effect on cell growth (Mendelsohn, 2000). The combination of CTX with some chemotherapeutic agents has been demonstrated in many human models, both *in vivo* and *in vitro*. There are studies showing that it increases the incidence of tumor cell apoptosis (Tortora *et al*., 1999; Bruns *et al*., 2000; Inoue *et al*., 2000).

Table 3. TUNEL results for all groups in a $1x10^4$ m^2 region following CTX application in 24 and 48 hours of incubation

	24h	48h
CONTROL		
0,1µg/mL CTX		
1 µg/mL CTX		
10 µg/mL CTX		
25 µg/mL CTX		

3.3. RT-PCR Analysis Results

While CDKs are involved in cell proliferation together with cyclins, CDK inhibitors cause cell cycle arrest by inhibiting the cyclin complex with CDKs (Cordoncardo, 1995; Maclachlan *et al*., 1995). The genes involved in the regulation of the G1 checkpoint in cancers are frequently mutated. In addition, changes and disruptions in the activities of these genes not only negatively affect the control of the cell cycle, but also affect DNA repair genes and especially the response of p53 to DNA damage (Li *et al*., 2005). The G1 to S phase transition pathway is frequently disrupted in human cancers, and the Rb protein is at the center of this pathway (Weinberg, 1995; Sherr, 1996). p21, p27, and p57 are members of the CDK inhibitor family, and they inhibit the phosphorylation of Rb protein by binding to CDK and cyclin complexes (Cyclin E-CDK2; Cyclin A-CDK 2; and Cyclin D-CDK 4) (Xiong et al., 1993; Polyak et al., 1994; Sherr, 1996). Solmi and coworkers (2008) investigated the effect of CTX on some genes in HT-29 and Caco-2 CRK cell lines and reported that CTX increased P53 in both cell lines. In addition, they

suggested that *p21* and *p57* were downregulated by CTX in Caco-2 CRK cells. In a study by Lim and coworkers (2007), it was observed that luteolin applied to HT-29 CRK cell lines decreased *p21* and *p27* protein levels. They observed that a concentration-dependent reduction of mRNA levels of *p21* was observed in the treatment of HT-29 CRC cell lines with an increasing concentration of luteolin. In another study, CTX was applied to the HepG2 hepatocellular cancer cell line and it was found that CTX inhibited the growth of the HepG2 cell line depending on the dose and time, and also increased the gene expression of *p21* and *p27*, which are CDKN inhibitors (Huether *et al*., 2005). In our study, a statistically significant increase in *p21, p27*, and *p57* gene expression was observed in all groups compared to the control group after 24 and 48 hours of CTX application (Table 4).

Table 4. *p21, p27* and *p57* gene expression levels of all groups at 24 and 48 hours

Genes		Control	0.1µg/mL CTX	1 µg/mL CTX	10 µg/mL CTX	25 µg/mL CTX
p21/G6PD	24h	0.010±0.0041	0.023±0.0052*	0.031±0.0032*	0.023±0.0021*	0.022±0.0029*
	48h	0.017±0.0051	0.019±0.0015	0.021±0.0010	0.033±0.0031*	0.027±0.0006*
p27/G6PD	24h	0.22±0.336	0.23±0.160	0.27±0.007*	0.28±0.006*	0.27±0.007*
	48h	0.251±0.002	0.270±0.004*	0.295±0.003*	0.270±0.004*	0.261±0.002*
p57/G6PD	24h	0.0210±0.0004	0.0279±0.0005*	0.0321±0.0003*	0.0290±0.0004*	0.0270±0.0073*
	48h	0.0299±0.0005	0.0520±0.0002*	0.0330±0.0004*	0.0410±0.0007*	0.0520±0.0008*

*It shows the statistical significance of comparing the groups in the same row with the control group ($p<0.05$).

KRAS protein, a proto-oncogene of the GDP family dependent on GTP/GTPase activity, participates in mitogenic signal transduction from the membrane to the nucleus. The KRAS protein is mainly involved in the transmission of extracellular signals of the MAPK pathway to the nucleus, which plays an active role in proliferation, differentiation, adhesion, migration, and apoptosis (Lievre *et al*., 2006). In our study, after 24 hours of CTX application, *KRAS* gene expression increased in all groups compared to the control group, and the highest increase was observed in the group administered 10 µg/mL CTX. After 48 hours of incubation, a decrease was observed in all groups compared to the control group. The highest reduction was observed in the group administered 10 µg/mL CTX (Table 5).

Table 5. *Kras* gene expression levels of all groups at 24 and 48 hours

		Control	0.1µg/mL CTX	1 µg/mL CTX	10 µg/mL CTX	25 µg/mL CTX
Kras/ G6PD	24h	$0.21.10^{-3}$±$0.17.10^{-3}$	$0.85.10^{-3}$±$0.57.10^{-3}$*	$1.10.10^{-3}$±$0.58.10^{-3}$*	$1.43.10^{-3}$±$1.34.10^{-3}$*	$1.10.10^{-3}$±$0.41.10^{-3}$*
	48h	$2.01.10^{-3}$±$0.10.10^{-3}$	$1.61.10^{-3}$±$0.88.10^{-3}$*	$0.68.10^{-3}$±$0.09.10^{-3}$*	$1.26.10^{-3}$±$0.08.10^{-3}$	$0.40.10^{-3}$±$0.21.10^{-3}$*

*It shows the statistical significance of comparing the groups in the same row with the control group ($p<0.05$).

Autophagy and apoptosis are related to each other and can affect each other at the molecular level. Based on this interaction, we examined the effect of CTX inhibition of EGFR on autophagic genes in the HT-29 CRC cell line. As a result of our studies, when different concentrations of CTX were applied to HT-29 CRC cells in 24 and 48 hour incubations and these values were compared with *LC3A* gene expression, an increase was observed in all groups compared to the control group. When we investigated the effect of CTX on *BECN1*, another autophagic gene, we observed that *BECN1* gene expression increased in all groups compared to the control group in 24 hours of incubation, and decreased in all groups compared to the

control group in 48 hours of incubation. According to these results, it can be said that CTX causes an increase in the expression of autophagic genes *LC3A* and *BECN1* (Table 6).

In the study of Feng and coworkers (2016), on the HT-29 CRC cell line, with the combination of CTX and 5-Fluorouracil (5FU), it has been suggested that 5FU induces autophagy by sensitizing cells. According to the results of western blot analysis, an increase was observed in LCI and LCII, which are the gold standard markers for autophagy. In the same study, it was stated that the use of CTX alone did not have a significant effect on cell death. A report on the basis of HT-29 and SW480 CRC cell lines demonstrated that *BECN1* was activated in cells in a dose-dependent manner when different concentrations of anti-EGFR were applied (Chen *et al*., 2016). In another study investigating how CTX affects the relationship between apoptosis and autophagy in A437, DiFi, and HN5 cell lines, it has been reported that apoptotic cell death occurs with the application of CTX to the DiFi cell line and it is said to induce autophagy to protect cancer cells from death. *LC3* gene expression was examined after CTX application in all cell lines and it was reported that the highest increase was in the DiFi cell line (Li *et al*., 2010). The *ATG4* protein, which is the target of Caspase 3, which plays an important role in apoptosis, is another protein that shows that autophagy and apoptosis are interrelated at the molecular level. When applying CTX to the HT-29 CRC cell line, *ATG4A* gene expression was increased in all other groups compared to the control group after both 24 and 48 hours of incubation (Table 6). Similar to our study, it was reported that the combination of cisplatin and melatonin applied at different concentrations to the HT-29 CRC cell line increased the autophagic genes *BECN1, ATG4A*, and *LC3A* gene expression (Polat, 2015).

Table 6. *LC3A, ATG4A* and *BECN1* gene expression levels of all groups at 24 and 48 hours

		Control	0.1µg/mL CTX	1 µg/mL CTX	10 µg/mL CTX	25 µg/mL CTX
LC3A/G6PD	24h	0.044±0.006	0.082±0.001*	0.106±0.003*	0.116±0.006*	0.067±0.004*
	48h	0.047±0.001	0.091±0.005*	0.067±0.007*	0.105±0.008*	0.104±0.004*
ATG4A/G6PD	24h	0.0145±0.0026	0.0330±0.0011*	0.0420±0.0007*	0.0420±0.0006*	0.0400±0.0007*
	48h	0.0230±0.0008	0.0570±0.0007*	0.0500±0.0002*	0.0660±0.0003*	0.0720±0.0011*
BECN1/G6PD	24h	0.110±0.005	0.140±0.002*	0.141±0.006*	0.150±0.005*	0.131±0.003*
	48h	0.160±0.008	0.140±0.008*	0.120±0.007*	0.150±0.008	0.160±0.004

*It shows the statistical significance of comparing the groups in the same row with the control group (p<0.05)

Epidermal growth factor (EGF) is a growth factor that plays an important role in cell growth, proliferation, and differentiation. It shows its effect by binding to its own receptor (EGFR). Factors such as EGF and TNF-α compete with CTX for binding to EGFR. For this purpose, the levels of *EGF* gene expression were evaluated by applying different concentrations of CTX to the HT-29 CRC cell line in the present study. Compared to the control group, an increase in *EGF* gene expression was observed after 24 hours of incubation in all CTX-treated groups, while a decrease was observed after 48 hours of incubation (Table 7). Tabernero and coworkers (2010) suggested that patients with CRC had a strong increase in TGF-α protein levels during monotherapy treatment with CTX and showed that this trend was also present in *EGF*.

Table 7. *EGF* gene expression levels of all groups at 24 and 48 hours

		Control	0.1µg/mL CTX	1 µg/mL CTX	10 µg/mL CTX	25 µg/mL CTX
EGF/G6PD	24h	$1.8.10^{-3}$±$0.8.10^{-3}$	3.32. 10^{-3}±$0.7.10^{-3}$*	3.97. 10^{-3}±$0.6.10^{-3}$*	4.74. 10^{-3}±$0.5.10^{-3}$*	3.36. 10^{-3}±$0.6.10^{-3}$*
	48h	$8.35.10^{-3}$±$0.63.10^{-3}$	6.43. 10^{-3}±$0.49.10^{-3}$*	6.52. 10^{-3}±$0.35.10^{-3}$*	8.34. 10-3±$0.64.10^{-3}$	7.16. 10-3±$0.71.10^{-3}$*

*It shows the statistical significance of comparing the groups in the same row with the control group (p<0.05)

4. CONCLUSION

In this study, the effects of CTX, an EGFR inhibitor and anticancer agent, on *p21, p27,* and *p57* genes, which are important in apoptosis, were investigated in the HT-29 CRC cell line. In addition, CTX's effects on autophagic *BECN1, ATG4A*, and *LC3A* genes, together with *KRAS* and *EGF*, which are important in cell proliferation, were also investigated. According to the MTT results, it was observed that CTX showed its best effect after 24 hours of incubation. In parallel with the literature, it can be said that CTX increases *p21, p27*, and *p57* gene expression in HT-29 CRC cells and also leads to apoptosis due to the increase in TUNEL positive cell density. At the same time, in light of these data, it is thought that CTX directs cells to autophagy by increasing *BECN1, ATG4A*, and *LC3A* gene expression in HT-29 CRC cells. On the other hand, when the effect of CTX on *EGF* and *KRAS* gene expression, which is involved in cell proliferation, is investigated, it is considered that CTX inhibits cell growth by reducing *EGF* and *KRAS* gene expression within 48 hours. While there are numerous studies on *KRAS* gene mutation in the literature, no study has been found that investigates the effect of CTX or other chemotherapeutic agents on *KRAS* gene expression.

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The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Fatma Betul Ozgeris: Experiment design, laboratory work, writing. **Mevlut Sait Keles:** Experiment design, supervision. **Eda Balkan:** Laboratory work, validation. **Adem Kara:** Laboratory work, validation. **Nezahat Kurt:** Statistical analysis.

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CRISPR/Cas9 and its Application in Plant Biotechnology

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Abstract

The clustered regularly interspaced short palindromic repeat-associated protein9 genome editing system (CRISPR/Cas9) was first discovered in prokaryotic organisms. Then, it was a widely used tool for eukaryotes. According to the other genome editing systems such as ZFNs and TALENs, CRISPR/Cas9 is much more preferred by researchers due to its simplicity and high efficiency. CRISPR /Cas9 vector systems have been developed for applying this technology to numerous plant species. In this study, we present a review of CRISPR/Cas9 system and its application in plant biotechnology.

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1. INTRODUCTION

Until the beginning of the 19th century, the world population was approximately one billion. Since then, growth rates have been increasing steadily more than necessary and are expected to increase nearly 12% more by 2050, and 25% more by the end of the century compared to now. Today, the world population has exceeded 7.8 billion. Due to this rate of population growth, it is predicted that the need for food will increase compared to previous years, and the available resources will be insufficient (Tilman *et al.,* 2011; Schultz, 2018; Steduto *et al.,* 2018; Wagh *et al.,* 2019). Food and agricultural products are the most important primary needs in human life. Plants are a major source of food for all living organisms and use energy from sunlight (Scheben *et al.,* 2017; Wagh *et al.,* 2019). In addition, plants are constantly attacked by numerous pests and pathogens such as parasitic plants, nematodes, bacteria, fungi, insects, and viruses. All of these organisms decrease agricultural production and yield in many parts of the world (Fisher *et al.,* 2012; Dangl *et al.,* 2013; FAO, 2016). Although conventional breeding methods have provided significant stages in controlling plant viruses, pathogens stress factors, most of these methods still have some problems, such as a lack of enough resources for disease resistance and stress factors. New genetic modification techniques improve disease-related traits from various species. Some of the modifications carried out have taken their place in the literature as endonucleases, which is a new technological approach. These endonucleases; "zinc finger nucleases" (ZFN) (Figure 1) (Maeder *et al.,* 2008; Langner *et al.,* 2018) "transcription activator-like effector nucleases" (TALEN) (Figure 2) (Boch *et al.,* 2009; Moscou and Bogdanove, 2009; Christian *et al.,* 2010; Bogdanove and Voytas, 2011; Langner *et al.,* 2018) and "clustered regularly interspaced short palindromic repeats" (CRISPR) (Jinek *et al.,* 2012; Cong *et al.,* 2013; Mali *et al.,* 2013a; Langner *et al.,* 2018). The development of genome editing called "zinc finger nucleases" (ZFN), "transcription activator-like effector

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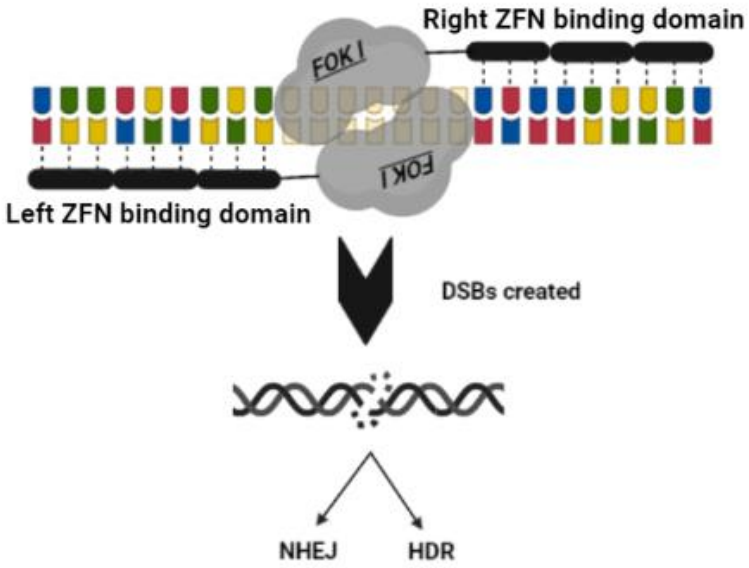
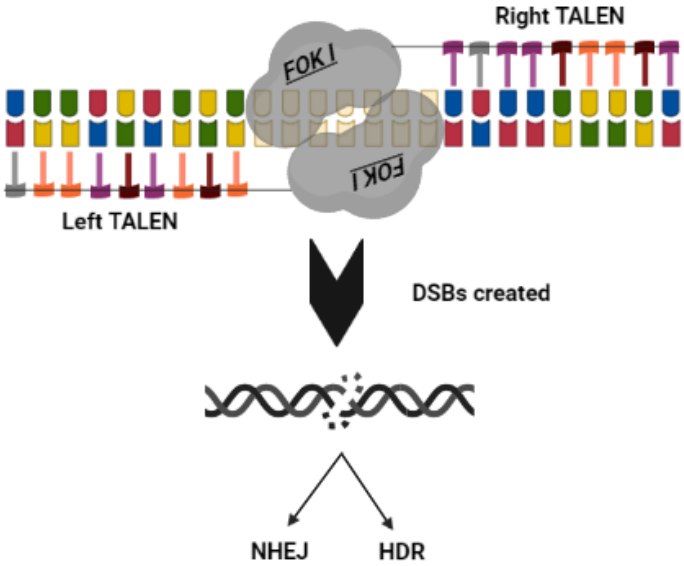
nucleases" (TALEN) and and "clustered regularly interspaced short palindromic repeats" (CRISPR) has created new opportunities in genome editing. The genome editing tools have been utilized successfully for the first target-gene of tobacco protoplast in 1998 (Paszkowski *et al.,* 1988) and increased with target gene activity from double-strand breaks (DSB) has been reported by many researchers (Puchta *et al.,* 1993). ZFN, one of the genome editing tools, can be used successfully to improve to yield of plant pathogens and environmental stress in many plant species (Guan *et al.,* 2002; Wright *et al.,* 2005; Shukla *et al.,* 2009). TALEN proteins belong to a central domain responsible for DNA binding, a nuclear localization signal, and a domain that activates the target gene transcription. Upon working principles and structures have been shown to similar activity, however; it has higher specificity than ZFN. ZFNs, sequence-specific nucleases fuse the specific DNA-binding domain of an artificial zinc finger sequence to the non-specific cleavage domain of the FokI (Figure 1). Three base pair (bp) sequences are targeted by ZNFs. Since FokI has a dimeric function, two ZFN monomers, 18-24 base pair sequence which has a 5-7 nucleotide (nt) spacer are designed, and ZFNs is acting as a site-specific nuclease cutting DNA (Chen *et al.,* 2019).

Figure 1. Illustration of ZFN (Created with BioRender.com)



TALE proteins are artificial enzymes derived from TAL effectors of *Xanthomonas* bacteria. The DNA-binding domain is shown to include monomers, each of the monomers binds one nucleotide in the sequence. Single DNA is tandem repeats of 34 amino acid sequences. These repeats are nearly identical except for two positions at amino acids 12 and 13, which define DNA binding specificity following a given code (Boch *et al.,* 2009; Moscou and Bogdanove, 2009; Boch and Bonas, 2010; Schornack *et al.,* 2013). This peerless characteristic of the TAL effector, the conglutinating of the unitary DNA-binding domain of the TAL effector to the catalytic domain of the FokI endonuclease (Figure 2), led to the design of TALENs. To design specific TAL sequences is easier than ZFNs and TALENs are capable of binding their intended DNA targets with 90% efficiency (Voytas, 2013). TALEN genome-editing tool was used in the regulation of EGL3 and KNAT of endogenous genes in *Arabidopsis* (Morbitzer *et al.,* 2010) and the regulation of *AtPAP1* gene in tobacco (Liu *et al.,* 2014). In 2013, various studies were conducted on rice, wheat (Shan *et al.,* 2013), *Nicotiana benthamiana* Domin (Nekrasov *et al.,* 2013) and *Arabidopsis thaliana* (L.) Heynh (Li *et al.,* 2013).

Figure 2. Illustration of TALEN (Created with BioRender.com)



The CRISPR-Cas9 genome editing system is discovered to study the defense mechanisms of some bacterial species (Barrangou *et al.,* 2007; Langner *et al.,* 2018). This newly developed technology has been used in healing studies on many organisms, especially plants (Li *et al.,* 2013; Langner *et al.,* 2018). With this genome editing system, specific arrangements have been made, making a great contribution to plant breeding, and this system has pioneered the production of efficient products. CRISPR is more likely to target than other genome editing tools, and is just as easy to work in the lab. Studies in plant species are generally focused on improving desirable properties, increasing yield, and developing resistance to various stress factors (Chen *et al.,* 2019). In recent years, studies have also been carried out in different areas such as the application of CRISPR in epigenetic mechanisms (Schaart *et al.,* 2021) and polyploid plants (Matres *et al.,* 2021). Using CRISPR, safer food production can be achieved by reducing or completely eliminating the damage caused by the transgene, and this can help the agricultural sector to progress (Chen *et al.,* 2019). This new technology is being implemented in a way that covers all disciplines and has created new perspectives in genome editing studies that are popular nowadays (Hsu *et al.,* 2014; Wagh *et al.,* 2019). In addition, Emmanuelle Charpentier and Jennifer Doudna recently won the Nobel Prize in Chemistry (in 2020) for their works on CRISPR and its technology for the development of the gene-editing tool. CRISPR/Cas systems are a unique function providing organisms protection in response to cellular immunity, Cas proteins and substrates have been shown different activities in the systems. In terms of the diversity of Cas proteins and the molecules, CRISPR/Cas systems are defined as six main types (type I, II, III, IV, V, and VI) and are grouped into two separate classes (ClassI and II). Class I CRISPR/Cas systems include Types I, III, and IV, while Class II CRISPR/Cas systems include types II, V, and VI. Class I CRISPR/Cas systems function with multiple Cas proteins, while Class II CRISPR/Cas systems operate with a single Cas protein (Makarova *et al.,* 2015; Makarova *et al*. 2018).

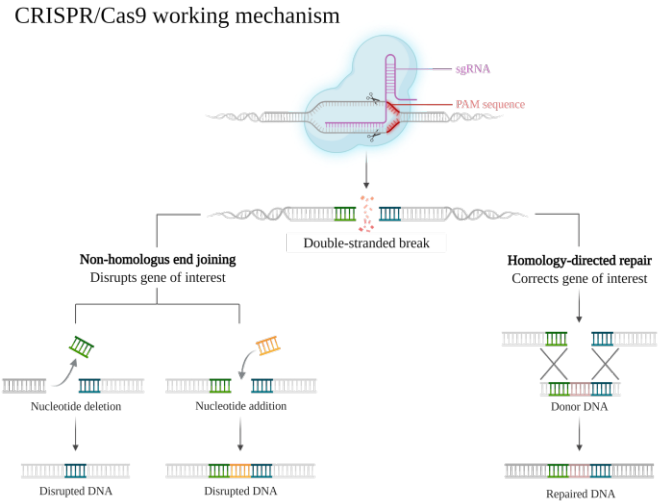
Type II CRISPR/Cas9 is the first characterized and a single effector protein found in the type II systems, and other CRISPR systems have been added to the literature later on (Zheng *et al,* 2020). Type VI CRISPR/Cas13 is the RNAi (RNA interference) of the type III systems, developing and detecting only RNA molecule shaping and manipulation tools (Cox *et al.,* 2017; O'Connell, 2019). CRISPR/Cas14 are central players in the type II systems, cleaving single-

stranded DNA (Harrington *et al.,* 2018). We review the function, mechanism, biology, and applications of the type II CRISPR systems.

1.1. How It Works the CRISPR/Cas9 System

The CRISPR/Cas9 system has two key elements. The first of these is Cas proteins and the second is CRISPR-RNAs. Cas9 enzyme, which is also an endonuclease, has the ability to cut a specific target site by using two short RNA molecules. These RNA molecules are tracrRNA (trans-encoded CRISPR-RNA) and crRNA (CRISPR-RNA) and can be artificially fused to form a chimeric RNA molecule called sgRNA (single guide RNA) (Mali *et al.,* 2013b; Qi *et al.,* 2013). sgRNA, which acts as an RNA-guided endonuclease that intercedes sequence-specific cleavage at the DNA, combines with Cas9 to form the sgRNA-Cas9 complex (Jinek *et al.,* 2012). Although that complex has a site-specific catalytic effect, it is defined by a sequence involving only sgRNA molecules (approximately 20 nucleotides). The design of 20 nt sgRNAs is quite simple compared to ZFNs and TALENs.

Figure 3. Schematic representation of the working mechanism of CRISPR/Cas9 (Adapted from "CRISPR/Cas9 Gene Editing", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates)



Genetic material is recognized and targeted by the Type II CRISPR/Cas9 system, which consists of a three-step process: acquisition, expression, and interference (Van der Oost *et al.,* 2009; Garneau *et al.,* 2010; Horvath and Barrangou, 2010; Marraffini and Sontheimer, 2010; Kumar and Jain, 2015). The first step (acquisition) involves the differential recognition and integration of foreign nucleic acid in the CRISPR locus. In general, the protospacer includes a short-conserved PAM (NGG) nucleotide sequence that serves as a recognition motif for DNA fragment acquiring (Figure 3). A spacer of about 30 base pairs (bp) is caused by the leader strand of the CRISPR sequence, and then this sequence is copied (Garneau *et al.,* 2010; Kumar and Jain, 2015). In the second step (expression) of the CRISPR/Cas9 system, long pre-crRNA (primary transcripts) is actively transcribed from the CRISPR locus and crRNAs are refunction with the help of Cas enzymes and tracrRNA molecule. In a recent study, it was reported that tracrRNA assists in the processing of pre-crRNA in *Streptococcus pyogenes* (Karvelis *et al.,* 2013). Through base complementation, the repeat region of crRNA pairs with tracrRNA and provides processing of pre-crRNA into crRNA. Processed crRNAs enter CASCADE (the CRISPR-associated antiviral defense complex) and help in base pair recognition with a specific target site of foreign nucleic acid (Deltcheva *et al.,* 2011). In the third step (interference), crRNA directs the Cas protein complex to the specific target site of the foreign nucleic acid

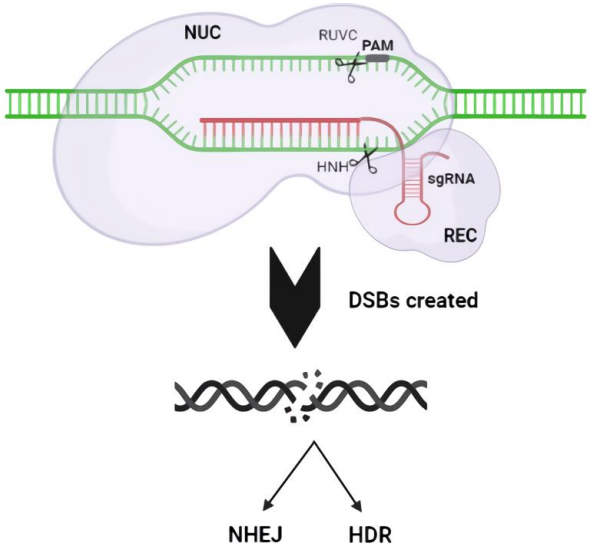
sequence for cleavage and initiates the cleavage of this region (Garneau *et al.,* 2010; Marraffini and Sontheimer, 2010; Kumar and Jain, 2015).

The CRISPR mechanism performs the editing process by creating breaks on the DNA double-strand (Voytas, 2013; Puchta, 2017; Langner *et al.,* 2018). Repair of these double-strand breaks is achieved by "homology-directed repair" (HDR) and "non-homologous end-joining" (NHEJ) systems (Figure 3). Although NHEJ is used more frequently, it has a high error rate as it causes insertions or deletions on the living genome compared to other repair systems (Lieber, 2010; Symington and Gautier, 2011; Knoll, *et al.,* 2014; Puchta, 2017). HDR, on the other hand, gives more accurate and reliable results (Puchta, 2005; Bortesi and Fischer, 2015; Langner *et al.,* 2018).

1.2. Cas9 Enzyme

Streptococcus pyogenes Cas9 enzyme is a multi-domain and multifunctional DNA endonuclease, which has 1368 residues. (Jiang and Doudna, 2017). It cuts 3 bp of dsDNA (double-stranded DNA) upstream of the PAM via an HNH-like nuclease domain, which cleaves the guide RNA's (gRNA) complementary DNA sequence, and a RuvC-like nuclease domain liable for cleaving the DNA strand across the opposite strand (Figure 4) (Gasiunas *et al.,* 2012; Jinek *et al.,* 2012; Chen *et al.,* 2014). In addition, the Cas9 enzyme also takes an active role in crRNA maturation and spacer acquiring (Heler *et al.,* 2015). When the crystal structure of Cas9 was examined, it was determined that it originate in two lobes, the first lobe is REC which recognizes and the second one is NUC (nuclease) lobe. The REC lobe contains three regions in the form of a long alpha-helix, also called the bridging helix, and REC1 and REC2 domains. The NUC lobe contains the PAM RuvC, HNH, and C terminal (CTD or PAM-interacting; PI) domains (Nishimasu *et al.,* 2014). Also, the PAM recognition site is unregulated in the Cas9 inactive structure.

Figure 4. Illustration of Cas9 enzyme with its subunits (Created with BioRender.com)



When the Cas9 enzyme is in the apo state, it cannot recognize the target DNA without binding to a guide RNA. Some reports show that apo-Cas9 attaches randomly to DNA and can swiftly cleave DNA regions nonspecifically at the existence of competitor RNAs. (Sternberg *et al.,* 2014; Jiang and Doudna, 2017). The structural overlap of apo-Cas9 along with sgRNA and DNA-bound structures indicates that this apo-Cas9 exhibits an inactive state in the apo-form and requires induced constitutive activation by RNA for recognition of DNA and cleavage it (Jiang and Doudna, 2017). This steric observation indicates that the nuclease construct of the

Cas9 enzyme is not active in the absence of associated gRNAs (guide RNA) (Jinek *et al.,* 2012). It also supports the view that Cas9 acts as an RNA-guided endonuclease (Jinek *et al.,* 2014). In another study, Cas9 without a guide RNA (gRNA) was sent to *Corynebacterium glutamicum* cells and it was observed that these cells did not form any colonies, which revealed that Cas9 without guide RNA adversely affected cell survival (Jiang *et al.,* 2017). In addition, overexpression of dCas9 (a catalytically dead Cas9) in *E. coli* caused cell growth to slow down, resulting in cell shape and structure disruptions. It has been reported that the toxicity of Cas9 is caused not only by DNA cleavage, but also by transient nonspecific DNA binding throughout the genome (Cho *et al.,* 2018). Furthermore, this system is dependent on the functional role of regulatory proteins fused to dCas, rather than on various repair mechanisms. These dCas regulatory fusion proteins are site-specific regulators of gene expression. Depending on the function of fused regulatory proteins, gene expression can either be activated (CRISPRa) or inhibited (CRISPRi) (Xu and Qi, 2019).

1.3. Formation and Interactions of Guide RNA

Cas9 makes extensive interactions with sgRNA. The Hel-I domain of Cas9 interacts in many ways with the arginine-rich bridging helix and the binding region between stem-loop 1 and stem-loop 2 (hairpin/hairpin loop), the anti-relapse duplex, and hairpin-1 via the CTD domain (Jiang and Doudna, 2017). In contrast, Cas9's primarily RuvC and CTD domains interact less extensively with the hairpin-2 region of sgRNA. Due to the absence of a 3' tracrRNA tail in the sgRNA structure, no protein-RNA interaction was observed for the hairpin-3 in the Cas9-sgRNA structure. Also, DNA target-bound structures show that Cas9 has little association with hairpin-3 (Nishimasu *et al.,* 2014; Jiang *et al.,* 2016). Biochemical studies have shown that sgRNAs lacking the linker region induce DNA cleavage, though, with less efficiency, the absence of hairpin-1 completely abolishes the cleavage (Jinek *et al.,* 2012). In light of these studies, it can be concluded that the anti-repetition duplex and hairpin-1 are irreplaceable for Cas9-sgRNA complex formation, whereas the linkers are not essential for function but can stabilize gRNA binding and promote complex formation activity (Nishimasu *et al.,* 2014; Jiang and Doudna, 2017).

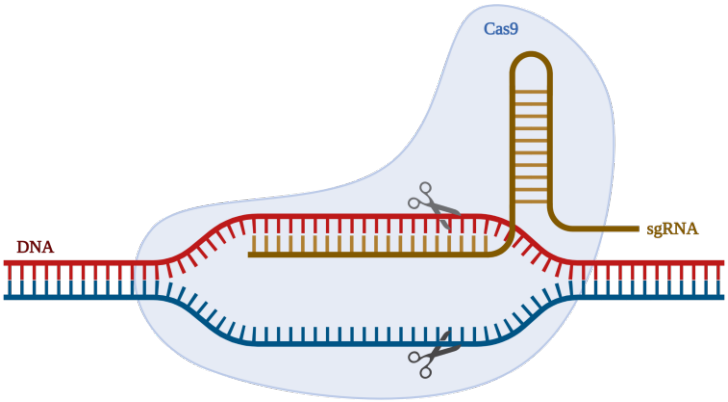
1.4. Target Searching and Recognition

When Cas9 binds the gRNA, it begins to search for complementary target DNA regions (Jiang *et al.,* 2015). Target seeking and recognition requires the presence of a conserved PAM sequence adjacent to the target site, as well as complementary base pairing between the 20-nucleotide (nt) target DNA's spacer site and a protospacer (Gasiunas *et al.,* 2012, Jinek *et al.,* 2012). The PAM sequences are very important structures for the distinction between sense and antisense sequences (Marraffini and Sontheimer, 2010), and point mutations in PAM inactivate Cas9's notch activity in vitro (Jinek *et al.,* 2012). One of the commonly used PAM motifs is 5'-NGG-3' belonging to SpyCas9, where N; it can be one of the bases adenine (A), thymine (T), guanine (G) or cytosine (C). Several assays have concluded that Cas9 starts the target DNA search process by searching for a suitable PAM site ahead of scanning neighboring DNA for possible gRNA complementarity (Sternberg *et al.,* 2014). Target recognition takes place via three-dimensional impacts due to complementariness between gRNA and adjacent DNA, by the time Cas9 swiftly cleaves from DNA lacking the appropriate PAM sequence and detects a suitable PAM sequence (Sternberg *et al.,* 2014; Knight *et al.,* 2015; Ma *et al.,* 2016). Upon encountering a target site containing the correct PAM, Cas9 initiates DNA lysis at the PAM-adjacent DNA nucleation site, followed by the occupation of the RNA sequence to form an RNA-DNA hybrid and a PAM-replaced DNA strand (Sternberg *et al.,* 2014; Szczelkun *et al.,* 2014). A perfect match between the center site of the sgRNA and the target DNA is crucial for Cas9's DNA targeting and cleavage, whereas defective base pairing(s) in the noncentral site can be tolerated for target binding specificity (Wu *et al.,* 2014; Jiang and Doudna, 2017).

1.4.1. RNA–DNA Hybrid

The final complex constructs of Cas9-sgRNA, whether they involve a partly duplexed PAM-containing DNA sequence, bind to complementary single-stranded target DNA. Electron micrograph of Cas9-sgRNA indicates that bind to perfectly paired target dsDNA including a canonical PAM motif (Anders *et al.,* 2014; Nishimasu *et al.,* 2014). These bindings provide great structural information about how Cas9-sgRNA recognizes its substrate, dsDNA. (Jiang *et al.,* 2016). Among them, there is a mechanism by which Cas9-sgRNA interacts with the target single-strand DNA (ssDNA), as first seen from the ssDNA-bound structure (Nishimasu *et al.,* 2014). In this conformation, the target DNA's strand hybridizes with the sgRNA's spacer sequence (Figure 5), resulting in an RNA-DNA hybrid with a skewed conformation, structurally more like an A form. The RNA-DNA hybrid is located in the central groove between the RECognize and NUClease lobes and is recognized by Cas9 in a sequence-independent manner. This indicates that Cas9 picks out a guide target heteroduplex form rather than the sequencing of nucleic acid. The A-form configuration of the RNA-DNA hybrid is preserved in the structure to which the PAM duplex is linked (Anders *et al.,* 2014). This event provides an interpretation of RNA-targeting by Cas9 in the existence of oligo DNA PAMmers. Additionally, as an RNA-RNA duplex it regularly has a similar A-form conformation. (O'Connell *et al*., 2014). Closer examination of superimposed RNA-DNA hybrids in all DNA-bound constructs, including ssDNA, PAM duplex, and dsDNA-linked constructs, especially from positions +12 to +17 towards the 5' end of the guide RNA, shows that the hybrid duplex exhibits more impairment (Jiang and Doudna, 2017).

Figure 5. RNA-DNA hybrid (Adapted from "CRISPR/Cas9", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates)



1.4.1. PAM Sequence Recognition

In this construct, a Cas9 nickase (H840A) forms a complex with a partially duplexed target DNA comprising 83-nt sgRNA and a canonical 5'-TGG-3'-PAM sequence on the non-target strand. This complex mimics a partly cleaved product containing a non-target and a target DNA strand. The PAM duplexes are located in a positive-charged groove between the REC and NUC lobes, and the PAM-containing non-target strand is found mainly in the CTD domain. The first base in the PAM sequence, denoted by the letter "N", remains paired with its corresponding base, but N and Cas9 do not affect each other. PAM's "GG" dinucleotides (guanine dinucleotide) can be read straight-forward in the main groove inherence specifically for base-hydrogen bonding interactions where residual of two arginines located in the beta-hairpin of the CTD domain. This direct base readout through hydrogen bond interactions provides better sequence specificity and larger discrimination compared to that observed by recognition at the minor groove of DNA (Rohs *et al.,* 2010; Jiang and Doudna, 2017). In

particular, Cascade recognizes the PAM sequence in a double-stranded form (Hayes *et al.,* 2016). Additionally, base-specifical contingence with GG dinucleotides, the CTD domain of Cas9 produces multiple hydrogen bonds interactions with the deoxyribose-phosphate backbone of the PAM-including non-target DNA. After all, no direct contact was observed between Cas9 and target nucleotides whose PAM is complementary (Anders *et al.,* 2014). This conformational finding indicates that Cas9 tolerates the PAM sequence in the non-target strand rather than the target strand, and mismatches of the PAM duplex in the target strand region (Jinek *et al.,* 2012; Sternberg *et al.,* 2014).

1.5. Cutting Target Area

With PAM recognition and RNA-DNA hybrid formation, Cas9 becomes active for making a notch in DNA (Sternberg *et al.,* 2014). Cas9 utilizes two nuclease domains, a fine-conserved RuvC domain including three divided RuvC motifs, and an HNH domain at the center position of the protein (Figure 4) (Jiang and Doudna, 2017). Each domain unbinds a strand of target dsDNA at a specific site from the 3 bp PAM sequence to produce a blunt-ended DNA double-strand break (Gasiunas *et al.,* 2012; Jinek *et al.,* 2012). However, Cas9 nickases cleave only one strand of DNA, causing a single-stranded break (Jinek *et al.,* 2012). When such Cas9 nickases targeting opposite strands are paired with sense/antisense sgRNAs, they produce staggered cuts in the target DNA so that Cas9 nickases can generate double nick-induced DSB for genome editing specificity (Ran *et al.,* 2013).

The binding of gRNA to Cas9 triggers a major conformational rearrangement, which converts Cas9 from an inactive form to an active form in DNA recognition (Jinek *et al.,* 2014, Jiang *et al.,* 2015). For target binding and strand invasion, the RNA sequence pre-converts to the A-form conformation and thus PAM-recognition sites become pre-located for PAM interrogation (Jiang *et al.,* 2015). Cas9's initial interaction with PAM sequences allows Cas9 to quickly scan the neighboring sequence for possible target sequences. (Sternberg *et al.,* 2014; Singh *et al.,* 2016). When Cas9 finds a proper target with PAM, it initiates bidirectional decoding and continues sampling the remain over target sequences (Sternberg *et al.,* 2014; Szczelkun *et al.,* 2014; Rutkauskas *et al.,* 2015). The phosphate lock residue stabilizes the separated strands of target DNA so that the first base of the target strand can rotate and translate towards the gRNA for pairing, and Cas9 interacts with the inverted bases on the non-target strand to enable bidirectional unbinding (Jiang *et al.,* 2016; Palermo *et al.,* 2016). gRNA target base pairing and structural alterations of Cas9 lead up to sgRNA strand invasion somewhere beyond the source site of DNA (Nishimasu *et al.,* 2014). The non-center site is successively separated from the restriction, and the base-pairing spread towards the 5' ends of the guide sequence (Sternberg *et al.,* 2014; Szczelkun *et al.,* 2014; Jiang *et al.,* 2016). This cascade base pairings causes more adaptive changes in Cas9 until it reaches the active status (Cencic *et al.,* 2014; Josephs *et al.,* 2015). Consequently, complete binding of gRNA and target DNA strand allows HNH to get a stable, steady and active form so that it can cleave the target site (Jiang *et al.,* 2016; Palermo *et al.,* 2016). Such conformational changes of HNH also result in a bigger conformational change in loop-linkers that direct the non-target strand to the RuvC catalytic center for proper cleavage (Sternberg *et al.,* 2015; Jiang *et al.,* 2016). Cas9 remains stringently bound to the fragmented target DNA until other cellular factors remove it (Sternberg *et al.,* 2014; Jiang and Doudna, 2017).

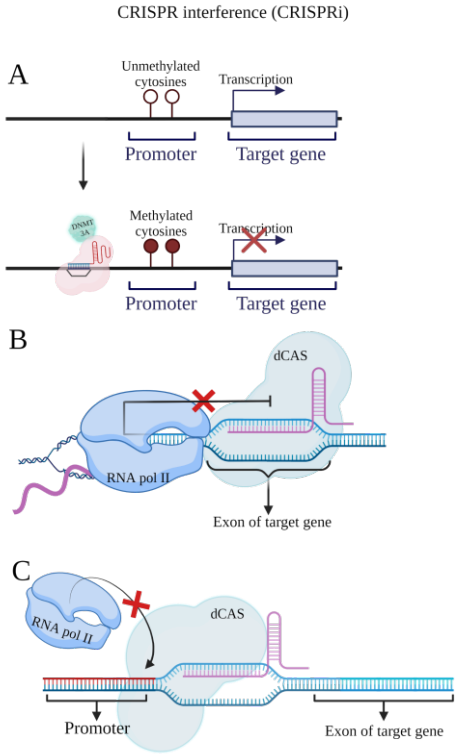
1.6. CRISPR/Cas9 Applications in Genome Editing

In 2007, Rodolphe Barrangou *et al.,* (2007) in their first article published in Science magazine; tested with CRISPR whether *Streptococcus*, a lactic acid bacterium, could be resistant to a phage. It has been observed that *Streptococcus*, when the spacer link is inserted, renders it resistant to the corresponding phage of this strain, and the resistance disappears when

the protospacer sequence that generates this bacterial resistance is silenced. In the study by Brouns and his team, it was noted that precursor RNAs from *E. coli* CRISPR were first synthesized (about 120-180 nucleotides), and then some of them were cut (about 60 nucleotides) with the activity of Cas genes and mature RNAs were formed. Again, in this study, van der Oost's group stated that Cascade, that is, the complex structure of Cas proteins, is one of the keystones for the antiviral immune mechanism, the formation of mature guide RNAs by the sub-union of CRISPR RNA endonucleases (Brouns *et al.,* 2008). Jillian Banfield and AF Andersson performed a metagenomic analysis on archaea to identify new gap sequences at CRISPR loci corresponding to phages in the same microbial communities (Andersson and Banfield, 2008).

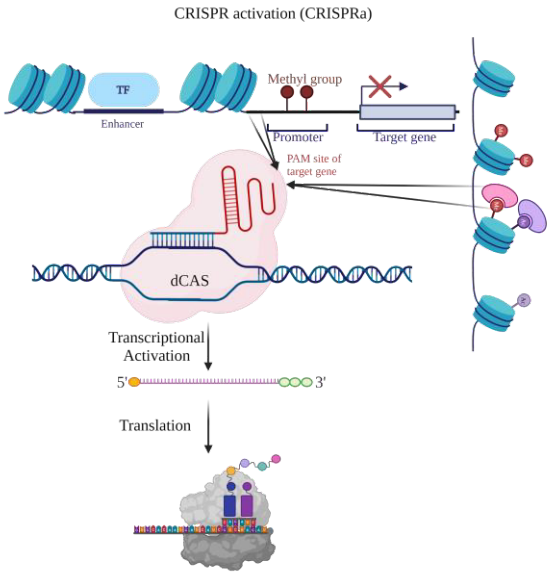
It was noted by Garneau *et al.,* in phage and plasmid studies of *Streptococcus thermophilus*, published in 2010, that Cas9 is the protein that can interfere with and cut DNA among Cas proteins. Evidence that Cas9 is the Cas protein required for CRISPR interference in the CRISPR/Cas9 system was demonstrated by Sapranauskas *et al.* (2011), when the *Streptococcus* CRISPR/Cas system was expressed in *E. coli*, demonstrating heterologous protection against plasmid transformation and phage infection of the reconstructed CRISPR system (Garneau *et al.,* 2010; Sapranauskas *et al.,* 2011). In addition, another study proved that the purified CRISPR/Cas9 RNA system can degrade target DNA in vitro (Gasiunas *et al.,* 2012). 2013 is a new beginning year for CRISPR and the CRISPR/Cas system of *Streptococcus* used CRISPR engineering for genome editing in mouse kidney cells and for mammalian cell studies of *Streptococcus thermophilus* and *Streptococcus pyogenes* (Cong *et al.,* 2013; Mali *et al.,* 2013b).

Figure 6. An image that describing the CRISPRi mechanism. **A:** Promoter silencing by dCas9-linked DNMT3A (DNA Methyltransferase 3 Alpha), **B:** Inhibition of elongation, **C:** Inhibition of RNA polymerase initiating. In all three cases, an editing/repression is done at the transcription level with dCas9 (Created with BioRender.com)



The catalytically dysfunctional, in other words, dead Cas9 endonuclease obtained as a result of point mutations in the endonuclease domains of Cas9 endonuclease is called dCas9. Similar to its unmutated form, this inactive enzyme has found use in molecular biology by forming a complex with the gRNA that has the PAM sequence. Because a mutation occurs only in the residue that displays endonuclease activity, dCas9 can still bind to gRNA and target DNA. Such binding properties and inactivation of Cas9 may enable gene activation or inactivation in the CRISPR system. If gRNA is designed to prevent RNA polymerase from binding to DNA, dCas9 can block or at least weaken transcription of the gene (Figure 6). Conversely, dCas9's ability to bind to DNA with high fidelity may also allow dCas9 to be used to activate a gene (Figure 7). Taghbalout *et al.,* (2019) connected TET1 to dCas9, transmitted it to the target DNA, and provided a successful transcriptional activation. In the field of using dCas9, the CRISPR system is named in two different ways as CRISPRa (CRISPR activation) (Gilbert et al. 2013) and CRISPRi (CRISPR interference) (Qi et al. 2013). Jensen and his colleagues (2021), also showed that CRISPRa and CRISPRi systems were used successfully in primary cells.

Figure 7. A summary diagram showing the operation of the CRISPRa system (Created with BioRender.com)



1.7. CRISPR/Cas9 Applications in Plant Biotechnology

The CRISPR/Cas9 system has been used for various purposes in many fields since its discovery. These include not only model plants consisting of *Arabidopsis*, but also economically important plant species. most of these studies were carried out to increase yield crops, development of biotic and abiotic stress factors, gene transformation. However, in recent years, specific applications including genome modifications have been made to manipulate specific traits in different plant species. Food security and climate change are the most important issue all over the world. The agricultural product is greatly threatened by the increased frequency of droughts, heavy rainfall, fluctuations in temperature, salinity, and insect pest attacks on major food crops. Genome editing technologies have the potential to help produce high-yield crops under conditions of biotic and abiotic stress. CRISPR/Cas9 is an effective method that allows the formation and development of targeted mutations in agricultural products, enabling the mutation of new alleles required to be achieved through breeding programs lacks exogenous genetic materials.

CRISPR/Cas9 technology was first used in *Arabidopsis thaliana* and *Nicotiana benthamiana* plants, since it is a model organism, and studies on these plants were aimed at understanding the functionality of the system and elucidating the mechanism. The mutation of the *PDS* (Phytoene desaturase) gene was generally targeted in these studies. The *PDS* gene is an important gene involved in the carotenoid biosynthesis pathway and is associated with many biosynthesis pathways (Wang *et al.,* 2009). It is one of the special genes of the phytol ring and plastoquinone formation in the structure of chlorophyll. For this reason, it has been widely used in CRISPR/Cas9-based genome editing studies to examine the functionality of the system and to elucidate the mechanism (Fan *et al.,* 2015; Nishitani *et al.,* 2016; Kaur *et al.,* 2018).

After successful studies with model organisms, this system has been tested in rice (*Oryza sativa* L.) and bread wheat (*Triticum aestivum* L.), which are the main food sources worldwide and have high economic value. 92% of the genome editing studies were carried out in rice consist of CRISPR/Cas9 based studies (Belhaj *et al.,* 2013). Some of the studies conducted are studies on the mutation of Acetolactate Synthase (*ALS*), branching enzyme IIb (*BEIIa*), mitogen-activated protein kinase (*MAP2*), and 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPs*) genes (Belhaj *et al.,* 2013; Zhang *et al.,* 2014).

Successful studies have been reported on the creation of new characters targeted with CRISPR/Cas9 technology in plant breeding, the production of resistant plants against biotic and abiotic stress factors, the inactivation of characters with undesirable characteristics, and especially on genes with unknown functions. The CRISPR system was used to create a heritable mutation in tomato, and formed the mutations of *SlPDS* gene albino tomatoes and obtained T0 the first mutant plants as an additional mutation and there to 83% homozygous and biallelic mutant T_0 in plants has been reported that seen with the same high rate. (Pan *et al.,* 2016). Again, mutation in the *PIF4* (phytochrome interacting factor, *SlPIF4*) gene was targeted in tomato (*Lycopersicum esculentum* L.) using CRISPR/Cas9 technology. The *PIF* gene is a gene belonging to the basic helix-loop-helix (bHLH) transcription factor family responsible for the regulation of phytochrome biosynthesis. As a result of the study, a mutation was created in the *PIF* gene. Chimeras were observed with mutations in plants (Pan *et al.,* 2016). Another study in tomatoes was directed to the mutation of the *NPR1* (*Nonexpressor of pathogenesis-related gene1, NPR1*) gene. There is limited genetic information on the role of the *NPR1* gene in plants, especially against abiotic stress. This gene is a gene that plays a role in the defense mechanism in pathogen-induced attacks, which is also a salicylic acid receptor. It has been reported that the *NPR1* gene, which became inactive as a result of the mutation created in the research, makes the plant sensitive to drought stress in tomatoes and reduces stomatal activity by stimulating the negative expression level, and therefore reduces the resistance to drought stress in tomatoes (Cai *et al.,* 2015; Li *et al.,* 2019). On the other hand, a mutation in the recessive eukaryotic translation initiation factor4E (*eIF4E*) gene was targeted in the study conducted in *Cucumis sativus* L. Using the CRISPR/Cas9 system, point mutations (Single Nucleotide Polymorphism, SNP) were created at the N′ and C′ terminals of *eIF4E*, and plants with the gene with the mutation were selected and developed. Resistance to "Zucchini Yellow Mosaic Virus (ZYMV)" and "Papaya Ring Spot Virus (PRSV-W)" infections was observed in the developed plants (Chandrasekaran *et al.,* 2016). In wheat, it is aimed to create mutations in some genes such as PDS, MLO, and NAC2. It has been reported that as a result of the mutation created in the MLO gene by using CRISPR/Cas9 technology in wheat, plants resistant to yellow rust disease were obtained (Wang *et al.,* 2014; Wang *et al.,* 2017). Again, the genomes of many plants have been modified using CRISPR technology. Among the modified plant species, *O. sativa* (Jiang *et al.,* 2013; Zhang *et al.,* 2014; Sun *et al.,* 2016), wheat (Zhang *et al.,* 2016), *Zea mays* L. (Svitashev *et al.,* 2016), sorghum (Jiang *et al.,* 2016), tomatoes (Čermak *et al.,* 2015; Pan *et al.,* 2016), potatoes (Butler *et al.,* 2015; Wang *et al.,* 2015), grapes (Malnoy *et al.,* 2016; Ren *et al.,* 2016; Wang *et al.,* 2016b), apple (Malnoy *et al.,* 2016; Nishitani *et al.,* 2016), sugar

orange (Jia and Wang, 2014), lemon (Jia *et al.,* 2017), poplar (Fan *et al.,* 2015), soybean (Michno *et al.,* 2015), flax (Sauer *et al.,* 2016), alfalfa (Michno *et al.,* 2015) and tobacco (Jiang *et al.,* 2013; Li *et al.,* 2013; Nekrasov *et al.,* 2013).

In studies conducted with the CRISPR system on *Arabidopsis*, tobacco, lettuce, and rice, Cas9 protein and sgRNA were directly sent to plant protoplasts in the presence of PEG (polyethylene glycol), and it was reported that up to 46% targeted mutagenesis could be performed (Shan *et al.,* 2015; Woo *et al.,* 2015). Ito *et al.,* (2015) successfully created mutations on the *RIN* gene in tomatoes using CRISPR/Cas9 in their study. Silencing of the *RIN* gene, which is related to fruit ripening, the extended shelf life in tomatoes. The CRISPR/Cas9 genome editing approach has also been applied to improve key traits in other monocotyledonous plant species. Five gRNA sets were designed to inhibit the expression of endo-N-acetyl-ß-D-glucosaminidase (ENGase) using different transformation methods such as particle bombardment and Agrobacterium-mediated in barley, and it was shown that the ENGase gene was silenced by the CRISPR/Cas9-based method (Kapusi *et al.,* 2017). Kim *et al.,* (2018) reported the CRISPR/Cas9 system in wheat protoplasts for stress-related genes, *TaDREB2* and *TaERF3*. Approximately 70% of protoplasts were successfully transfected and expression of these upregulated genes was validated by T7 endonuclease assay. The CRISPR method has also been used in polyploid plant species. Hexaploidy potato genotypes, which are of great agricultural importance and economically valuable, were obtained by mutation of the GBSS (granule-bound starch synthase) gene using the CRISPR/Cas9 method (Jaganathan *et al.,* 2018). Similarly, multi-allelic mutagenesis was obtained by mutating *StALS1* in potatoes (Butler *et al.,* 2016).

Producing genetic variation in plants is one of the pillars of sustainable agriculture and disease management strategies (Griggs *et al.,* 2014). With the developing technology, the genome and transcriptome sequences of many plants have started a new era in plant breeding (Borrelli *et al.,* 2018). The genome editing technique is one of the recently popular biotechnological approaches in plant breeding. With the utility of genome editing technology in plant breeding, potential harmful allele formation due to hybridization and recombination is avoided and it is possible to directly improve genotypes (Zhang *et al.,* 2014). Plants are affected by different abiotic and biotic stress factors throughout their lives (Zaidi *et al.,* 2016). These stress factors are affected at the physical, biochemical, and molecular levels of plants and cause negative effects such as a decrease in yield and quality, abnormalities in growth and development, and deterioration in plant physiology. Therefore, it is of great importance for sustainable agriculture to protect plants from abiotic and biotic stress factors. Increasing the resilience of plants against diseases is important to meet the nutritional needs of the constantly increasing human population (FAO, 2017; Demirel *et al.,* 2020). The approach to control the disease using resistant varieties is often a useful method in agriculture. However, due to the high evolutionary potential of most plant pathogens, it is always possible to break the resistance present in plants. When a new strain of the pathogen, emerging by different mechanisms such as recombination or mutation, breaks disease resistance in resistant plants, existing disease management strategies can quickly become ineffective due to the increased frequency of the new genotype through natural selection and its rapid spread to other locations (Borrelli *et al.,* 2018; Demirel *et al.,* 2020).

Waxy maize enriched with amylopectin was developed by DuPont Pioneer by inactivating the endogenous *Wx1* gene responsible for amylose production. The flowering time of *Setaria viridis* (L.) P. Beauv. was delayed by inactivating the homolog of the *ZmID1* gene (Jaganathan *et al.,* 2018). The omega-3 fat content of the cayenne plant is increased. In the soybean plants, on the other hand, *Drb2a* and *Drb2b* genes have been inactivated and resistance to drought and salt stress has been gained (Scheben and Edwards, 2018).

There are studies on increasing biotic and abiotic stress resistance as well as crop yield by genome editing with the CRISPR/Cas system (Ricroch *et al.,* 2017; Tufan and Keleş, 2019). Wang *et al.,* (2020) reported that regulating the C-terminal region of cytokinin activation enzyme LOGL5 in rice, increased yield and increased resistance to various environmental conditions in rice. Similarly, in another previous study, Zhang *et al.,* (2019) obtained a high-yielding wheat phenotype by knocking out the cytokinin oxidase/dehydrogenase enzyme in wheat. In addition, Yuste-Lisbona *et al.,* (2020) reported that they can increase tomato size and yield with the CRISPR method by editing the *ENO* gene, which controls meristem size in tomatoes. Gao *et al.*, (2020), in their study, developed new waxy maize cultivars from 12 waxy maize with damaged *GBSS1* genes using the CRISPR/Cas system and reported that these cultivars have superior field characteristics. In the same year, Xu and his colleagues reported that they produced rice with low amylose content by creating various amino acid sequences as a result of base-specific changes with the same gene (*GBSS1*), again using the CRISPR method (Xu *et al.,* 2021). However, while the rice with low amylose content is of great importance for the industry, in terms of human health, rice with higher amylose content has also been developed, thanks to the targeted mutation of the starch branching enzyme using the CRISPR system, as in the study conducted by Sun *et al.*, (2017). In addition, in another study published by Dong *et al.* (2020), targeted gene insertion was performed in rice using the CRISPR/Cas system, and rice with enriched carotenoid content was developed.

The necessity of developing new strategies to fight phytopathogenic fungi has become a constant focus of researchers. Today, efforts to develop new strategies for resistance to fungi by targeting *R* and susceptibility genes (*S* genes) in the host plant have become popular thanks to CRISPR/Cas technology (Borrelli *et al.,* 2018; Demirel *et al.,* 2020). It has been determined that 16 *MLO* (mildew-resistance locus O-1) genes identified in tomato and *SlMLO1* gene among these genes play a vital role in susceptibility to powdery mildew (Zheng *et al.,* 2016). A 48 bp (base pair) deletion was created by selecting two different point targets on the *SlMLO1* gene using two sgRNAs with CRISPR/Cas9 technology. The new variety was named "Tomelo" and shown to be resistant to *Oidium neolycopersici* L. Kiss. (Nekrasov *et al.,* 2017). Additionally, CRISPR/Cas technology has been used by *Erysiphe necator* Schwein (Malnoy *et al.,* 2016), *Botrytis cinerea* Pers. (Wang *et al.,* 2018), *Blumeria graminis* f. sp. *Tritici* (Bgt) (Zhang *et al.,* 2017) and *Magnaporthe oryzae* (Wang *et al.,* 2016a) were used to obtain resistant plants against pathogenic fungi (Demirel *et al.,* 2020).

Plant pathogenic bacteria are heterotrophic organisms that can grow on the host plant, such as parasites. These bacteria cannot enter directly through plant tissue and infection occurs through natural openings or wounds such as stomata and hydathodes. After infection, basic symptoms such as necrosis, burns, cancer, wilt, and tumor development in the plant (Sobiczewski, 2008). In the fight against bacterial diseases, methods such as biological control and cultural measures are applied, as well as the use of some antibacterial compounds and antimicrobial peptides. In addition, stimulation of the systemic acquired resistance (SAR) system in plants has had great success in the prevention of disease in patho-systems such as bacterial cancer disease, bacterial wilt, and fire blight (Maxson-Stein *et al.,* 2002; Pradhanang *et al.,* 2005; Sen *et al.,* 2015). Apart from the SAR system, with the stimulation of ISR, another induced resistance system found in the plant, *Xanthomonas oryzae* pv. *oryzae*, *Pseudomonas syringae* van Hall, resistance against different bacterial pathogens such as *Ralstonia solanacearum* (Kloepper *et al.,* 2004). After silencing of some of the *SWEET* genes in rice as a result of mutations in the promoter, *X. oryzae* pv. *oryzae* bacteria has been developed (Oliva *et al.,* 2019; Xu *et al.,* 2019). Similar to these studies, by targeting the promoter of the *LOB1* gene in lemon, *X. citri* subsp. *citri*. The results of the resistance development study against citri bacteria have also been reported (Peng *et al.,* 2017). Zhang *et al.*, (2017), reported that

resistance against wheat rust mold developed after the mutation of 3 homologous enhanced disease resistance-1 (*TaEDR1*) genes in *T. aestivum* with the CRISPR system.

Phytopathogenic viruses, which have an important place among biotic stresses, cause a 10-15% decrease in the yield of agricultural products worldwide every year (van Regenmortel and Mahy, 2009). Some of this loss can be prevented by increasing the resistance of the host plant against plant viruses. Control of plant viruses often depends on the use of pesticides against virus-carrying vectors, but such an approach has many adverse effects on the environment (Bragard *et al.,* 2013). Although plant viruses are combated with the use of resistant plants, the diversity of viruses, their rapid evolution, and their transport to plants, mostly by insect vectors, render approaches to control plant virus diseases ineffective (Hanley-Bowdoin *et al.,* 2013). Therefore, it is of great importance to develop new strategies in the fight against plant viruses. Advances in genome editing are also offered new possibilities for increasing endurance in agricultural products. The CRISPR/Cas system, which has been popular in recent years, has been applied in plants to provide resistance to both biotic and abiotic stress (Arora and Narula, 2017). Different CRISPR/Cas strategies have been used to effectively interfere with eukaryotic DNA or RNA virus genomes (Price *et al.,* 2015; Price *et al.,* 2016). The CRISPR/Cas system is used to provide resistance to viruses in plants with two different approaches. In the first approach, virus replication is prevented by targeting the episomal or viral genome integrated into the plant genome.

In addition to CRISPR systems, the only base change possible adding deaminase has been shown in published articles, and it has been reported that base changes of adenine (A:T>G:C) and cytosine (G:C>A:T) are possible using the CRISPR+deaminase system (Komor *et al.,* 2016; Gaudelli *et al.,* 2017).

The cytosine base editing (CBE) technique, on the other hand, made a genetic modification by targeting only the cytosine base in wheat and rice (Shimatani *et al.,* 2017; Zhang *et al.,* 2017). Li *et al.,* (2018) used Cas9-adenosine deaminase to convert the AT base pair to GC base pair in wheat and rice, and in the same year, Kang *et al.,* (2018) demonstrated this in *Arabidopsis*. Additionally, Jin *et al.,* (2020) reported more specific gene editing in rice using the human *APOBEC3B* cytosine base editor. With the combination of these two systems acting on both bases simultaneously (A and C), Li *et al.,* (2020) showed that targeted random mutations can be generated in plant genes, thereby increasing herbicide resistance in rice. Wu *et al.* (2021), reported that they used the CRISPR/Cas system successfully in their study and showed that the "ASP_LSL" phenotype emerged as a result of the change (G→A) of the 521st amino acid of the gene located in an area of 212 kb between RM22445 and RM22453 on the short arm of chromosome 8 in rice. It has been reported that this phenotype causes decreased yield, seed retention rate, and the number of grains per cluster in rice, and as a result of the phylogenetic analyzes made by the researchers, it was emphasized that this gene and its mutations have high similarity in *Ralstonia solanacearum*, *Zea mays* and *Sorghum bicolor*.

Zhang *et al.* (2021), in their study in *Arabidopsis*, successfully was created a CRISPR-mediated mutation of the *GALT2-6* (5 genes in total) (Hyp-O-galactosyltransferases) genes, which adds galactose, the first sugar, to arabinogalactan-proteins (AGP), which is responsible for the response to salt stress and intercellular hormone transmission and growth-related mechanisms. In addition, in their article published, *Jung et al.* (2021), showed that there are two controls on the flowering mechanism in Chinese cabbage (*Brassica rapa* L.) using CRISPR/Cas9 systems and reported that there is a negative feedback loop between nitrogen signaling genes (*BrNIA1 – BrNIR1*) and *BrSOC1* (SUPPRESSOR OF OVEREXPRESSION OF CONSTANCE 1).

4. CONCLUSION

Although CRISPR/Cas9 can be applied to plant genome editing, there are still challenges over minimizing off-target rates, elucidating the system, and optimizing Cas9. Given its simplicity, flexibility, versatility, and efficiency, functional genomics and crop trait improvements are likely to be made largely using CRISPR/Cas9 technologies.

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Author Contribution Statement

Emre Ilhan: Writing, Editing, Validation**. Ayse Gul Kasapoglu:** Writing, Editing. **Selman Muslu:** Writing, Editing. **Muhammet Macit:** Writing. **Busra Sezer:** Writing. **Aleyna Mevlutogullari:** Writing. **Dilara Guler:** Writing. **Melisa Aydin:** Writing. **Feyza Eksi:** Writing. **Murat Aydin:** Writing, Editing, Validation.

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